abstracts: poster presentations

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Actin and Actin-Binding Proteins in Evolution and Development

P1

Assembly Dynamics of Archaeal Actin-related Proteins (ARPs) in *Schizosaccharomyces pombe*

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Actins are one of the most conserved proteins among eukaryotes. Actins have now been discovered in several bacterial and archaeal species. However, actin-like proteins from these bacterial and archaeal species are very divergent from those found in eukaryotes. The origins of eukaryotic actins have thus remained unclear until the recent discovery of a group of Archaea called the Asgard archaea. Metagenomic analyses have revealed that actins from phylum Lokiarchaeota belonging to the Asgard group are the closest relative of eukaryotic actins. Lokiarchaea encodes for an actin and two actin-related proteins (Arp). While it has been recently shown that profilin-like proteins from these archaea can interact with the human actin, the assembly and dynamics of Loki actins remain unexplored. We had earlier used the fission yeast, *S. pombe* to study the dynamics of bacterial and archaeal cytoskeleton proteins. Here we thus tested if actin and actin-related proteins (ARPs) from Lokiarchaea assemble into structures in fission yeast. We tagged Loki actin and Loki actin-related proteins (ARPs) to GFP at the N-terminus and expressed them as GFP fusions from the medium strength thiamine repressible promoter in fission yeast cells. While the Loki actin showed only diffuse fluorescence, both the Loki ARPs assembled into speckles and spots. Further, these patches were resistant to latrunculin-A treatment, showing that these assemblies of Loki ARPs were independent of the *S. pombe* actin. These results suggest that the Loki ARPs can assemble into very short dynamic filaments when expressed in eukaryotic cell. Future studies in different genetic mutants relating to actin function and co-localisation studies with *S. pombe* actin patch components should reveal the interactions of Loki ARPs, if any with its eukaryotic counterparts and homologues.

P2

The FHOD-family formin in *Caenorhabditis elegans* promotes striated muscle growth and dense body organization in a cell autonomous manner

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Formins are known regulators of actin and microtubule networks. Loss of mammalian formins, like the FHOD-family formin, FHOD3, in mice leads to embryonic lethality that occurs due to lack of myofibril
maturation during cardiac development, but its explicit role is unknown. The striated body wall muscles of *Caenorhabditis elegans* are a simple model system with well-characterized sarcomeres that have many vertebrate protein homologs. The availability of viable formin mutant worms and formins’ subtle effect on muscle development makes the body wall muscle, an excellent system to investigate the precise roles of formins. Previously, we observed the deletion mutants for two formin genes, *fhod-1* (homolog of FHOD3 gene) and *cyk-1*, developed thin muscles with abnormal dense bodies/ sarcomere Z-lines. However, the nature of the *cyk-1* mutation required maternal CYK-1 expression for the viability of the examined animals. In this study, we tested the effects of complete loss of CYK-1 using a fast-acting temperature-sensitive *cyk-1(ts)* mutant. Surprisingly, neither post-embryonic loss of CYK-1 nor acute loss of CYK-1 during embryonic sarcomerogenesis led to any muscle defects, suggesting CYK-1 might not play a direct role in muscle development. Consistent with this, examination of *cyk-1(Δ)* mutants re-expressing CYK-1 in a mosaic pattern showed CYK-1 cannot rescue muscle defects in a muscle cell autonomous manner, suggesting muscle phenotypes caused in *cyk-1(Δ)* mutants are likely due to secondary effects. Conversely, mosaic re-expression of FHOD-1 in *fhod-1(Δ)* mutants promoted muscle cell growth, as well as proper Z-line organization, in a muscle cell autonomous manner. As we can observe no effect of loss of any other worm formin on muscle development, we conclude that FHOD-1 is the only formin that directly promotes striated muscle development in *C. elegans*.

P3

**Rapid diversification of Arp2 specialized for roles in *Drosophila* sperm development**

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**Rapid diversification of Arp2 specialized for roles in *Drosophila* sperm development**

The actin cytoskeleton, which is composed of force-generating polymers, often forms branched networks that are critical in many fundamental cellular processes, including cell motility, cell division and vesicular movement. Branched actin networks are generated by the Arp2/3 complex, a 7-membered protein complex including actin-related proteins (Arps) 2 and 3. Similar to actin and most Arps, Arp2 is evolutionarily ancient among eukaryotes and under stringent sequence conservation, yet we surprisingly discovered two clade-specific gene duplications of Arp2 in *Drosophila*: Arp2D in the *obscura* clade and Arp2D2 in the *montium* clade. Our targeted sequencing and phylogenetic analyses of Arp2D and Arp2D2 show these duplicates have evolved independently and arose 13-14 million years ago at the origin of their respective clades. The two duplicates exhibit distinct sequence diversification from canonical Arp2, and their retention throughout evolution of the *obscura* and *montium* clades suggests functional importance. Unexpectedly, we found both duplicates are testis-specific in expression, whereas canonical Arp2 is ubiquitously expressed. Why would evolution select for a divergent Arp2 for roles in fly sperm development? To explore the function of a testis-specific Arp2, we focused on one duplicate and generated *D. pseudoobscura* flies expressing GFP-tagged Arp2D. We found it localizes to post-meiotic actin structures known as actin cones, which are critical in the uniquely syncytial nature of germ cell development in male insects. Fly germ cells remain interconnected within a cyst, and during the last step of sperm maturation, actin cones separate the sperm by translocating from the nucleus down the length of the tail, encasing each sperm in its own membrane and pushing out excess cytoplasm. We found Arp2D localizes to the front of actin cones, where branched actin networks actively extrude excess membrane and propel cones down the sperm tail. We also discovered Arp2D is
rapidly evolving, a phenomenon that is often spurred by strong selective pressures for more competitive sperm. Based on the recurrent diversification of Arp2 and the localization and positive selection of one duplicate, we hypothesize lineage-specific diversification of Arp2 in *Drosophila* has specialized actin polymerization for the construction of actin cones, ultimately generating more competitive sperm.

P4

**A rapidly evolving actin-related protein localizes to germline cytoskeletal structures for roles in *Drosophila* male fertility**

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Cytoskeletal proteins perform many fundamental biological processes in all eukaryotes and are highly conserved among most species. However, we have found that actin-related proteins (Arps) have undergone recurrent genetic innovation via gene duplication and positive selection (accelerated amino-acid substitutions) in both *Drosophila* and mammals. Unlike the well-conserved members of the Arp superfamily, divergent Arps appear to be testis-specific in expression, yet the function of a male gametic Arp in any species is unknown. Based on their recurrent emergence and rapid evolution, we hypothesized that testis-specific Arps play important roles in sperm production or fitness. To test this hypothesis, we investigated the function of Arp53D, a rapidly evolving Arp in the *D. melanogaster* testis. *Arp53D* is present in all *Drosophila* species, suggesting it performs a crucial function. Through cytological analyses, we found that Arp53D localizes to two critical germline-specific cytoskeletal structures in the testis: fusomes and actin cones. The fusome is an actin-enriched membranous organelle that connects all germ cells in a cyst in male meiosis. Actin cones are unique structures that separate syncytial spermatids post-meiosis into individual cells and push excess cytoplasm to the end of the sperm flagellar tail. We found Arp53D uniquely localizes to the leading edge of actin cones, and its non-canonical N-terminal tail is both necessary and sufficient for this localization. Based on these findings, we expected that loss of *Arp53D* would lead to a reduction in male fertility, yet surprisingly, Arp53D knockouts (KO) exhibit increased male fertility, which is more pronounced under conditions of stress. We discovered *Arp53D* KO sperm are shorter than wildtype sperm, suggesting elongation defects, and surmise that shorter sperm allow for increased sperm production or storage, and thus more progenies. To address why *Drosophila* species would harbor a gene that appears to lower male fertility, we examined whether a fitness cost is associated with lack of *Arp53D* and found that the KO allele is readily outcompeted by the wildtype *Arp53D* allele in a population, suggesting *Arp53D* has been retained in all *Drosophila* species for a fitness advantage. Given our findings for Arp53D’s unique localization and impact on fertility, we hypothesize that recurrent specialization of Arps among many species may serve cytoskeletal functions in males and play important roles in promoting proper sperm elongation.

P5

**Septins and a formin have distinct functions in anaphase chiral cortical rotation in the C. elegans zygote.**

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Many cells and tissues exhibit chirality that stems from the chirality of constituent proteins and polymers. For example, the C. elegans zygote undergoes an actomyosin-driven chiral rotation in which the entire cortex is displaced circumferentially around the division plane during anaphase. This phenomenon thus relates to how force and chirality are translated across scales. Although it is known that actomyosin contractility drives this rotation, the molecular mechanisms transmitting contractility to chiral movement, and dictating handedness, are not understood. Septins are candidates for contributing to cell-scale chirality due to their ability to anchor and organize the actomyosin cytoskeleton. Here, we show that septins are required for anaphase cortical rotation. In contrast, the formin CYK-1, which we found to be enriched in the posterior in early anaphase, is not required for cortical rotation, but contributes to its chirality. Simultaneous loss of septin and CYK-1 function led to highly abnormal and often reversed cortical rotation. Ongoing work addresses septin localization following CYK-1 depletion and F-actin organization following septin depletion. We propose that anaphase cortical contractility is biased in a chiral fashion via interaction between the circumferential cytokinetic ring and perpendicular, longitudinal formin-based actin bundles that have accumulated torsional stress during formin-based polymerization. This model is based on mechanistic insights into chirality from work with adherent mammalian cells [Tee et al. (2015) Nat. Cell Biol. 17(4), 445-457. doi: 10.1038/ncb3137]. Our findings thus shed light on the molecular and physical bases for cellular chirality in the C. elegans zygote. We also identify conditions in which chiral rotation fails but animals are developmentally viable, opening avenues for future work on the relationship between early embryonic cellular chirality and animal body plan.

P6

Molecular evolution of lasp family proteins

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In mammals, five proteins with nebulin repeats have been reported (nebulin, nebulette, N-RAP, lasp-1, and lasp-2) that comprise the nebulin superfamily. The smallest members of the nebulin superfamily are lasp family proteins, which consist of lasp-1 and lasp-2 in vertebrates and lasp in invertebrates (reviewed by Grunewald et al., 2008). Lasp-1 is expressed in various nonmuscle tissues and is localized in actin-rich subcellular regions. Lasp-2 is highly expressed in the brain and localized in filopodia and lamellipodia of neuronal growth cones. Lasp-2 is also localized in Z-lines and intercalated discs of striated muscle. LIM domain and the first nebulin repeat of lasp-2 has been reported to have actin-binding activity. SH3 domain of both lasp-1 and lasp-2 interact with component of focal complexes and bind to dynamin and control membrane transport. The expression pattern of ascidian lasp suggested invertebrate lasp proteins have similar functions to those observed in both vertebrate lasp-1 and lasp-2 (Terasaki et al., 2008). In this study, the evolution of genes of invertebrate lasp, vertebrate lasp-1 and lasp-2 was analyzed as follows: BLAST searching, comparison of exon-intron structures and synteny analysis. Proteins with nebulin repeat have not been found in plants, yeast, and slime mold. Capsaspora, which is thought be an origin of multicellular animals, has a lasp protein with LIM domain, a nebulin repeat, and SH3 domain. Phylogenic analysis showed actin-binding region and SH3 domain were well-conserved in all lasp family proteins in vertebrates (mammalians, avians, amphibians, and bony fishes) and invertebrates (ascidians, amphioxi, nematodes, arthropods, cnidarians, sponges, and capsaspora), but linker sequence of invertebrate lasp proteins are varied between phyla. We also confirmed nematode
lasp has actin-binding activity. Exon-intron structures of genes of lasp, lasp-1 and lasp-2 are conserved and lasp-2 genes of bony fishes and amphibians share the exons with nebullette in the same manner as avians and mammalians reported previously (Terasaki et al., 2006). Syntenic relationship between ascidians lasp, vertebrate lasp-1 and lasp-2 of were conserved. These findings suggested that the first lasp protein may have been generated through the combination of exons coding LIM domain and nebulin repeats, and consequently obtained actin-binding activity. Linker sequences were varied during diversification of invertebrate organisms. Lasp-1 and lasp-2 are thought to have been generated by gene duplication and each linker sequence was well-conserved in vertebrates and have universal functions.

P7

**Divergent Actin and a Lamellipodium-Like Structure in *Giardia***

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The tight conservation of eukaryotic actin underscores the fundamental role that the protein plays in maintaining cell shape and forming cellular structures. Actin controls these processes for the unicellular, intestinal parasite *Giardia lamblia*. However, the actin sequence from *Giardia* is the most divergent identified in eukaryotes to date, and small molecules that target actin are ineffective in *Giardia*. Additionally, the parasite lacks all canonical actin-binding proteins, many of which are essential in other eukaryotes. This suggests that actin in *Giardia* avoided the evolutionary constraints faced by other eukaryotes, thereby providing a natural experiment to probe fundamental actin functions. The divergence also makes actin and its regulatory network a potential therapeutic target in *Giardia*, which infects over 300 million people annually, with 20% of those cases resistant to front-line treatment. Current treatments for *Giardia* target all anaerobic organisms in the gut, which decimates the commensal flora of patients and leads to other pathologies, including irritable bowel syndrome. Using fluorescent microscopy, we visualize actin in the ventrolateral flange, a membrane protrusion resembling a lamellipodium involved in attachment by *Giardia*. Further, knockdown of actin decreases both the width of the flange and attachment by *Giardia* to a surface. We have begun biochemical and cryo-electron microscopy studies to explore how the large amount of variation in actin from *Giardia* affects actin filament structure, as well as electron cryotomography studies to examine the cytoskeletal architecture of the flange. Identifying functional properties that distinguish the actin cytoskeleton of *Giardia* from its vertebrate homolog will provide opportunities for new drugs that specifically target the parasite without harming the host. This work will also generate fundamental insights into core actin properties.

P8

**The PAM-1 aminopeptidase interacts with cytoskeletal regulators in controlling cortical dynamics in the one cell *C. elegans* embryo**

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Reorganization of the actomyosin cytoskeleton of the one-cell *C. elegans* embryo is necessary to set up the anterior-posterior axis. Polarity is established in part through cortical flow that requires numerous cytoskeletal proteins to control cortical dynamics. PAM-1 encodes a puromycin-sensitive
aminopeptidase required for proper regulation of the cortex. Our objective is to determine the mechanism by which PAM-1 regulates the cytoskeleton. *pam-1* mutants display phenotypic defects consistent with a misregulation of the actin cytoskeleton. First, protein localization of moesin is slower to clear from the posterior than in wild-type embryos. Second, non-muscle myosin, NMY-2 appears to have a less robust network of puncta around the cortex and is slower to clear from the posterior than in wild-type embryos. In wild-type embryos, both moesin and NMY-2 puncta are spread throughout the cortex and after polarization is initiated, they localize to the anterior side of the embryo and concentrate at the cleavage furrows of diving cells. Lastly, *pam-1* mutants show defects in the regulatory processes leading up to cytokinesis, such as an over-active cortex, weak or no pseudocleavage, and blebbing at the cleavage furrow. These defects suggest the cortical cytoskeleton is not properly regulated and could be due to other cytoskeletal regulators interacting with PAM-1 to regulate cortical dynamics. Anillin, encoded by the *ani-1* gene, functions by remodeling the actin cytoskeleton. It binds non-muscle myosin and is suggested to be a key regulator alongside PAM-1 in organizing the cytoskeleton. By using RNAi knockout of *ani-1* in both wild-type and *pam-1* mutants, we are working to propose a model for the interaction between the two proteins in preventing cortical defects and in organizing NMY-2 into puncta. We are also interested in understanding the phenotypes of *pam-1* mutants in improper organization of moesin and NMY-2 around the cortex, through using fluorescence tagging. These studies will help us to understand the required components and regulators at play in polarity establishment and cortical flow phenotypes in the *pam-1* mutants. This work is funded by NIH 2R15GM110614-02.

**P9**

**The role of ATX-2 and VPR-1 in sperm positioning within the *C. elegans* meiotic embryo**

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The role of ATX-2 and VPR-1 in sperm positioning within the *C. elegans* meiotic embryo Fertilization occurs during female meiosis in most animals, which raises the question of what prevents the sperm body (DNA, centrioles, and organelles) from interacting with the meiotic spindle. In *C. elegans*, the meiotic spindle and sperm body are maintained in opposite thirds of the ellipsoid zygote despite vigorous cytoplasmic streaming. In a previous study (Panzica et al. 2017. J Cell Biol 216: 2273), the sperm body was relatively stationary in control cells but moved long distances with the yolk granules when actin was depolymerized, resulting in sperm DNA within 2 µm of the meiotic spindle. This result led to the idea that the sperm body is anchored at the site of fertilization by cortical actin while maternal organelles move freely with cytoplasmic streaming. Simultaneous live imaging of paternal mitochondria, maternal ER and maternal yolk granules, however, revealed episodes with these organelles moving together. We are currently exploring the possibility that contacts between the maternal ER, paternal mitochondria, and cortical F-actin may play a role in restricting the movement of the sperm body to the posterior end of the embryo. Depletion of ataxin 2 (ATX-2) has been reported to disrupt ER organization (del Catillo et al. 2019. Traffic 20: 436) and VAPB (VPR-1) has been implicated in tethering both mitochondria and endosomes to the ER (Phillips and Voeltz. 2015. Nat Rev Mol Cell Biol 17: 69). We found that depletion of ATX-2 caused both incorrect positioning of sperm DNA and scattering of paternal mitochondria within the zygote. In contrast, the sperm body was incorrectly positioned within the embryo upon depletion of VPR-1, but the paternal mitochondria remained in a tight cluster around the paternal DNA. We are currently generating degron-tagged alleles of atx-2 and vpr-1 for rapid depletion to test how directly they are involved in correct sperm positioning inside the embryo.
Cancer Therapy: Defining Therapeutic Targets and New Therapeutics 1

P10

**Generation of multiple 3D cancer spheroids for high-content screening applications in heterogenous tumor cells.**
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Compelling evidence suggests that cancer cells grown in 3 dimensions (3D) mimic *in vivo* tumors better than cells grown in monolayers. As such, there is a growing interest in adapting 3D cell models especially in cancer research and drug discovery. Spheroids, cluster of cells grown in 3D, resemble solid tumors in their tissue architecture with an outer cell proliferation zone, a middle quiescent cell layer, and an inner hypoxic, necrotic core. This structure creates a gradient of nutrients and oxygen in the spheroid, which is important for assessing drug penetration effects and dose responses in a tumor. It is believed that cell-based assays using spheroids as cancer models will have better predictive responses to compound treatments compared to using cells grown in 2D. Another challenge in the design of advanced cell-based assay for cancer biology is addressing tumor heterogeneity. Tumors exhibit different extent of heterogeneity which contributes to acquired drug- and chemo-resistance. The ability to design more effective, personalized treatment will require drugs to be tested in multiple combinations from a tissue sample which has heterogenous cell population. The goal of this study is to develop a method to assess multiple tumor micro-tissues derived from a single sample that will be suitable for studying effects of anti-cancer-drugs as well as clonal heterogeneity and clonal development of tumors. We combined tissue culture of multiple spheroids with compound screening to assess cell viability of each spheroid in response to treatment. Based on our earlier work, we used an optimized one-step staining method and high-content imaging. More importantly, we increased the number of spheroids that can be generated by using 96-well culture plates with arrays of microwells patterned inside each well. We developed a 3D image analysis procedure to quantify spheroid size, morphology and cell viability using multiple descriptors including cell counts, diameter, and other characterizations in 3D, such as volumetric readouts, cell content and morphology. We demonstrated concentration-dependent responses for selected cytotoxic drugs, and evaluated cell responses to drugs using conventional cell lines as well as primary tumor samples. We developed imaging and analysis methods for multiple readouts to evaluate EC50s for different compound effects. Finally, a library of 119 approved anti-cancer drugs was screened at high and low concentrations and morphological evaluation of spheroid phenotypes was used to compare the potency of compound effects. We anticipate that this approach will have applications in drug discovery, clonal analysis of cancer cells, tumoroid research, and have the potential for further development of personalized medicine for cancer treatments.

P11

**Development of in vitro models for evaluating CAR-T cell therapy against solid tumors**
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Chimeric antigen receptor (CAR) immunotherapy is one of the most promising modern approaches for the treatment of cancer. Administration of CAR-T cells to treat solid tumors has long been seen as one of the most difficult therapeutic tasks. The first two clinical trials conducted in sarcoma and neuroblastoma patients showed clinical benefits of CAR-T cells, yet many hurdles still remain on the way to accessible and efficient therapy. In our study we estimated the efficacy of CAR-T cell therapy against in vitro models of prostate adenocarcinoma. CAR-T cells were produced from normal T cells using 2nd-generation anti-CD19 CAR construct with built-in GFP reporter. As the model object we chose PC-3M-luc tumor cell line transduced with lentiviruses encoding for Katushka red fluorescent protein and CD19 antigen. The respective PC-3M-luc(Kat+) and PC-3M-luc(Kat+CD19+) cells were grown as 2D monolayer and 3D multilayer assemblies (using Inkredible bioprinter, Cellink). Upon application of CAR-T cells the number of PC-3M-luc(Kat+CD19+) reduced significantly, whereas no substantial alteration was observed for PC-3M-luc(Kat+) control. The expected mechanism of CAR-T cell cytotoxicity involved perforin/granzyme-mediated apoptosis. The antitumor efficacy was confirmed by means of fluorescent (Axio Imager 2, Zeiss) and confocal (LSM 780, Zeiss) microscopies - for 2D and 3D models, respectively. Overall, our results support the long-term promise of CAR-T cells as an efficient and safe therapy against solid tumors. Work was funded by RSF grant 19-74-20026 to A.R. A.V. was supported by personal stipend of the President of Russian Federation CNI-227.2019.4. References Titov, et al. "Advancing CAR T-Cell Therapy for Solid Tumors: Lessons Learned from Lymphoma Treatment." Cancers 12, 1 (2020): 125. DOI: 10.3390/cancers12010125

P12

Development of macromolecule-based drug delivery system nanoparticles for lung cancer therapy
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In the US, lung cancer is the second most common cancer and the leading cause of cancer mortality. Many chemotherapeutic agents are being used in clinic for decades, however, the main problem is the low therapeutic index and the acquired resistance to the drugs (e.g. Cisplatin and Doxorubicin). In this way, the use of macromolecules and lipids as the drug carriers, as targeting ligands or as therapeutic agents has had great impact in the development of drug delivery system nanomedicines. The aim of this research project is the development of drug delivery systems (DDS) using biocompatible macromolecules, i.e. bovine serum albumin (BSA), Lysozyme (Lyz) and methyl-β-cyclodextrin (mβCD) as the drug's carriers. Doxorubicin (DOX), Berberine (Ber) and curcumin (Curc) were conjugated to the carriers using oil in water in oil (O/W/O)-like emulsion system followed by heat and ultrasonication. The DDS have three drugs incorporated in the carrier structure by non-covalent interactions. To characterize the ratio of the conjugation of these systems colorimetric assays were performed. DDS's size was determined ~ 100 nm using dynamic light scattering. All the developed DDS demonstrated an IC50 in the μM range after 24h incubated with lung cancer cells (A549). A complete discussion of the results will be presented. These DDS have potential to minimize drug systemic toxicity and increase drug bioavailability. Future experiments will be performed to determine the impact to reduce multidrug resistance syndrome.
De novo pyrimidine biosynthesis maintains ribosomal RNA production and nucleolar morphology in glioblastoma.

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Metabolic alterations leading to increased synthesis of nucleotides by de novo pathways are emerging as key alterations driving glioblastoma growth. Glioblastoma is the most frequent type of malignant brain tumor; its poor prognosis is often marked by recurrence and resistance to chemotherapeutic agents such as temozolomide. In this study, we demonstrate that glioblastoma cells activate de novo pyrimidine biosynthesis to support the high production rate of ribosomal RNA. Inhibition of the de novo pyrimidine biosynthesis pathway with the DHODH inhibitors brequinar or ML390 depletes the pool of pyrimidines, thus impairing the synthesis of ribosomal RNA and leading to aberrant nucleolar morphology as a sign of nucleolar stress. Moreover, brequinar and ML390 treatment decreases the proliferation of glioblastoma cells, including temozolomide-resistant cells. Our study identified an approach to inhibit ribosome production and consequently the proliferation of glioblastoma cells through the specific inhibition of the de novo pyrimidine biosynthesis pathway.

Notch signaling contributes to proliferation of glioma cells via the regulation of Hexokinase2

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Notch signaling, which plays important roles in cell-cell communication, promotes cancer proliferation including glioma cells. However, some types of gliomas are reported to be resistant to Notch inhibitors (Gamma-Secretase Inhibitors, GSIs). We previously found that Notch signaling negatively regulates gene expression of Hexokinase2 (HK2) in glial cells of zebrafish embryos (Notch-HK2 inhibitory pathway). Among glycolytic enzymes, HK2 is known to be involved in cell proliferation. Therefore, we hypothesized that Notch-HK2 inhibitory pathway mediates GSIs resistance of gliomas. In this study, we explored some conditions and factors necessary for the activation of Notch-HK2 inhibitory pathway. We evaluated HK2 expression levels in U87 cells (Human glioma cell line) which were exposed to GSI under various nutrient conditions. As a result, GSI did not affect HK2 levels compared to DMSO controls when U87 were cultured in the normal medium of EMEM + 10% FBS. On the other hand, HK2 was increased by GSI treatment under the condition that glucose and serum were removed from normal medium (GS-free condition). In addition, U87 cells were co-cultured with Notch ligand expressing cells in order to identify which ligand especially activates Notch-HK2 inhibitory pathway. Under the GS-free condition, Notch activation due to Delta-like1 (Dll1) and Jagged1 induced the decrease in HK2 whereas Dll4 ligand did not. Furthermore, to assess whether HK2 inhibition by Notch also occurs in the in vivo environment, we established a xenograft model of zebrafish in which U87 cells were transplanted in the brain. In comparison with the results of in vitro experiments, the increase of HK2 expression by GSI exposure was
more significant in U87 transplanted embryos. We also examined the influence of Notch inhibition on the viability of gliomas using live-cell imaging method. Consistent with previous studies, the viability of U87 was similar between the control group and the GSI-exposed group in xenograft embryos. Taken together, our findings suggested that glioma cells activate Notch-HK2 inhibitory pathway depending on the nutritional state and the type of Notch ligands, which may lead to low sensitivity to GSIs.

P15

Phagocytosis of Metastatic Tumors in CD47 Immune Checkpoint Blockade

J. C. Andrechak

Phagocytosis of Metastatic Tumors in CD47 Immune Checkpoint Blockade


A common model of solid tumor metastasis involves tail-vein injection of a cancer line which then lodges within the lung vasculature and subsequently grows, but interactions with circulating and resident immune cells remain unclear. Mouse melanoma-derived B16F10 cells are used here to generate poorly immunogenic, syngeneic tumors in C57BL6/J mice in order to assess an emerging macrophage checkpoint pathway. CD47 is a ubiquitously expressed membrane protein that signals ‘self’ to the macrophage receptor, SIRPα, ultimately inhibiting phagocytosis - at least when a phagocytic target is also opsonized by antibody to activate macrophage Fc-Receptors. Blockade of CD47 shows some initial clinical success in combination therapy against liquid tumors although off-target phagocytosis of blood cells is at least a safety concern and perhaps undermines therapy. CRISPR-Cas9 knockout of CD47 in B16F10 (KO) enables the study of tumor-opsonizing antibody therapies in the absence of CD47 inhibitory signaling. B16F10 lung tumor-bearing mice can also be treated with anti-Tyrp1 mAb, which is specific to a melanocyte surface antigen that engages and activates Fc-Receptor mediated phagocytosis by macrophages. In vitro studies indeed show that anti-Tyrp1 mAb increases phagocytosis of KO cells relative to unopsonized KO cells or opsonized wild-type cells (WT). Lung metastatic nodules of KO cells grow exponentially similar to WT tumors, and untreated mice succumb in ~3 wks. Anti-Tyrp1 infusions significantly suppresses growth of KO but not WT tumors, indicating the necessity of the combination, and growth suppression prolongs survival by up to 100%. Histology suggests immune infiltration within lung tumor nodule borders of treated mice moreso than untreated. In vitro phagocytosis of normal blood cells abrogates on-target phagocytosis of KO cells, suggesting that off-target effects in the clinic might also significantly hinder therapy. To thus minimize blood cell phagocytosis that is widely reported with systemic anti-CD47, we did tail-vein injections of engineered marrow macrophages with SIRPα blockade plus anti-Tyrp1 (to specifically target the B16 cells); these cells suppress tumor growth roughly similar to the KO plus anti-Tyrp1. Thus, given the proper combination of activating cues and checkpoint block, metastatic tumors can be effectively engulfed to prolong survival.

P16

Modeling and Deciphering COJEC Resistance Using Neuroblastoma PDX Models

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Intense multi-modal treatment for high-risk (HR) neuroblastoma (NB) has significantly increased overall survival, but resistance and relapses are common and side effects are severe. One critical step is rapid COJEC induction, consisting of cycled administration of Cisplatin, Vincristine, Etoposide, Cyclophosphamide and Carboplatin. COJEC has been used in the clinic for many years, but a comprehensive preclinical study investigating the mechanisms of COJEC resistance had not been done. The aims of this project are: 1) to establish a clinically relevant COJEC protocol with NB patient-derived xenograft (PDX) models; 2) to analyze the mechanisms of COJEC resistance; and 3) to establish tumor organoids from COJEC-resistant PDX tumors. MYCN-amplified HR-NB PDXs were established in nude and NSG mice and treated with a COJEC-like protocol for 6 weeks. Several dose regimes were tested and treatment was personalized depending on tolerance. Responsive tumors were subjected to surgical resection and observed for relapse. All tumors were analyzed using chromosomal copy number analysis, RNA sequencing and immunohistochemistry. Fresh PDX tumors were dissociated to establish COJEC-resistant organoids. HR-NB PDXs treated with a COJEC-like schedule presented responses comparable to those seen in the patients from which they derive, ranging from significant increased survival to poor or no response. Considerable tumor volume reduction was observed for one PDX model, with a 23% relapse rate after surgical removal. Data from this responsive PDX model showed that treated tumors displayed a more differentiated histological profile, with high levels of key extracellular matrix components and increased cell death. Surprisingly, substantial cell proliferation was still observed during treatment, pointing to a possible population of resistant cells. Moreover, treated tumors presented an accumulation of chromosomal aberrations, with sub-clonal dynamics enhanced in relapsed tumors. Additionally, preliminary data from tumors that did not relapse showed a downregulation of genes associated with cell cycle and DNA replication/repair at the moment of surgical removal. This significant downregulation can also be observed in patients that did not relapse. Finally, various PDX tumors were dissociated and successfully cultured in stem cell medium as organoids, providing a useful in vitro tool. Preliminary results show that organoids derived from relapsed tumors are more resistant to COJEC drugs than those derived from untreated controls. We have established and optimized a clinically relevant COJEC induction protocol using HR-NB PDX models that will help study COJEC resistance. Additionally, our COJEC-resistant organoids are a useful tool for testing novel therapeutics in HR-NB.

P17

Actin-binding protein Profilin1 is a novel interventional target in clear cell renal cell carcinoma

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Clear cell renal cell carcinoma (ccRCC), the most common subtype of renal cancer, has a poor clinical outcome. In this study, we performed analyses of The Cancer Genome Atlas (TCGA) transcriptome data to demonstrate that increased mRNA expression of actin-binding protein profilin1 (Pfn1), along with several other key regulators of actin cytoskeleton including Arp3, coflin1, Ena/VASP and CapZ, that are
indicators of poor prognosis in ccRCC. Immunohistochemistry-based classification of Pfn1 staining in tissue microarrays indicated Pfn1-positivity in both tumor and stromal cells; however, the vast majority of ccRCC tumors tend to be Pfn1-positive selectively in stromal cells only. This finding is further supported by evidence for dramatic transcriptional upregulation of Pfn1 in tumor-associated vascular endothelial cells (VEC) in the clinical specimens of ccRCC. Patients with ccRCC also exhibit increased levels of soluble Pfn1 in the serum when compared to normal donors. In vitro studies support the importance of Pfn1 in proliferation and migration of RCC cells, and in soluble Pfn1’s involvement in VEC-tumor cell crosstalk. Finally, by computationally guided biochemical screening of small molecules, we identify a novel Pfn1-actin interaction inhibitor and provide proof-of-concept for the ability of this inhibitor to reduce proliferation and migration of RCC cells in vitro and tumor growth without eliciting any toxic effects in vivo. Based on these findings, we propose a potentiating role for Pfn1 in promoting tumor cell aggressiveness, with Pfn1 also serving as a potential interventional target in the setting of ccRCC.

P18

**A family-wide computational analysis of the catalytic domains of the Never-in-mitosis A-like (NEK) protein kinases**

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The human Never in Mitosis A-like kinase (NEK) protein family are serine/threonine kinases that have been functionally implicated in the regulation of the disjunction of the centrosome, the assembly of the mitotic spindle and the function of the primary cilium. Protein kinases that regulate the centrosome cycle are often aberrantly controlled in tumor cells. NEK2A, for example, is frequently up-regulated in multiple types of human cancers. To fully exploit NEKs as therapeutic targets, we need to understand their structure-function relationship and establish the foundation of their mechanism of action. In this study we present a detailed computational analysis of all eleven NEK proteins. Three-dimensional modeling of the catalytic domains, using various approaches including homology modeling, threading, and *ab initio* techniques was performed, followed by *in silico* protein-protein predictions and docking analysis. Our analyses show unique features present in different NEK family members, such as the armadillo (ARM)-like fold in NEK 10 and the RCC1 repeats found in NEK 8 and NEK 9. We predict unique protein-protein interactions that have not been previously reported such as NEK7 and TNF. These findings are corroborated by our docking analysis that shows predicted interaction via hydrogen bonds and salt bridges in the E-F LOOP area of TNF and specific residues of NEK 7. Overall, this study provides novel and intriguing information about the NEK protein family, unique protein-protein interaction prediction, and lays the groundwork for elucidating their molecular mechanism to investigate their potential as drug targets.

**Cell-Cell Interactions**

P19

**Postembryonic Invasion of Skin-derived Ionocytes into Hair Cell-containing Mechanosensory Organs**

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Embryonic organ morphogenesis often requires cells to migrate long distances, coalesce and re-arrange in a spatiotemporally controlled manner. However, reports of postembryonic migratory events that alter the cellular composition of pre-existing, functional organs are scarce. Here, we report the discovery of Neuromast-associated ionocytes (Nm ionocytes), a previously uncharacterized cell type that invades mature mechanosensory organs of the zebrafish lateral line postembryonically. This process, that we call ‘Programmed Organ Invasion’, is dynamically regulated by changes in environmental stimuli, such as pH and salinity. Using high-resolution in vivo time lapse imaging and zebraweb lineage tracing, we characterize the translocation of these highly motile, skin stem cell-derived cells and show that they enter the sensory organ as individual, closely associated pairs of cells. After extensive re-arrangement of the cells within the sensory organ that is accompanied by the extension of highly dynamic cellular protrusions, they anchor in a stereotypical position, in close association with mechanosensory hair cells. In fact, 3D reconstruction of Nm ionocyte ultrastructure by serial block face electron microscopy revealed the formation of a microvilli-containing apical crypt in close vicinity to the hair cell bundles and in direct contact with the hair cell microenvironment. Molecular analysis of these cells by scRNAseq and loss of function approaches show that Nm ionocytes maintain the proper ionic composition surrounding lateral line hair cells in response to changing aquatic environments. In sum, this study provides a detailed characterization of cells invading a mature sensory organ postembryonically. The possibility to trigger and modulate this invasive process by external stimuli, as well as the accessibility of the lateral line organ system to live cell imaging approaches makes Nm ionocytes an excellent model to study cell migration and invasion in unprecedented detail.

P20

A puncture would model for dynamic cell differentiation in a constrained space

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A puncture wound may well be the oldest known human health hazard. As a developmental system, it is a relatively underappreciated. Damage evoked chemical and mechano-chemical stimuli trigger resting platelets to multiple activation states that are organized to produce bleeding cessation within minutes. We present evidence from 3D visualization of 1000s of progressive SBF-SEM and correlative light microscopy on patterning platelet activation state to define thrombus structure. Results -- One min post puncture, platelets were anchored in clumps along the exposed vessel wall. Near the vessel wall was a peripheral layer of activated or degranulated platelets covered by additional layers of less-activated platelets. Aggregates of platelets with a mixture of activation states were found extending points into the hole and vertically into the intravascular space. Less than 40% of neighboring platelets were of the same activation state as their neighbor and <2% of platelet-occupied volume within the puncture hole contained largely degranulated platelets. At 5 min after injury, the puncture hole was sealed on the extravascular side by a platelet cap while on the intravascular side the hole was open to the vessel.
lumen. We conclude that bleeding cessation is due to extravascular capping of the puncture hole rather than to hole infill. Vertical platelet aggregates (columns) containing a mixture of platelets in different states extended intravascularly into the vessel lumen. Columns were typically lined on their surfaces by highly degranulated platelets and were spatially separated by large cavities, that we term vaults. Most platelets were weakly activated as indicated by morphology. At 20 min post-puncture, the intravascular surface of the thrombus was covered with an ~10 platelet-thick layer of loosely packed, variably activated platelets. The interior of the thrombus was filled with a mixture of tightly packed platelets that surrounded central, vertical aggregates of degranulated platelets. Vaulting was very limited in extent and restricted to central portions of the thrombus appeared to be spatially compressed, presumably due to associated fibrin accumulation as indicated by immunofluorescence.

Conclusions: Our results demonstrate dynamic spatial patterns of platelet activation within a forming puncture-wound thrombus. Such patterns raise novel questions as to how a combination of chemical signals and cell-cell interactions produce this outcome. The overall structure of the thrombus was complex and what the organizing principles are that produce these outcomes are not obvious. We hope that such results encourage interest from cell biologists in thrombus formation as an example of spatially constrained differentiation by a single cell type, the anucleate platelet.

P21

Investigating principles of homeostatic vascular remodeling via 4D imaging of live mice
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Our organs depend on an organized vascular network that supplies them with nutrients and soluble factors via the bloodstream. The endothelial cells (ECs) that line our blood vessels dictate crucial aspects of vascular function and morphogenesis. However, there are still many open questions concerning the cellular mechanisms by which the vasculature is able to establish and maintain homeostatic conditions. The challenge in addressing these questions is the inability to follow the same tissues and cells over time. To overcome this roadblock, we have established an intravital imaging approach to track and manipulate the same ECs in live mice to ask how vessel remodeling is orchestrated as a vascular plexus is established and the mechanisms in place to maintain vascular homeostasis. Longitudinal imaging of vessel remodeling of the dermal vascular plexus in neonatal mouse skin reveals that vessel regression significantly outpaces new vessel growth (angiogenesis) during postnatal development, and that vessel regression is accelerated in mutant mice lacking the actin cytoskeletal remodeler Rac1 in ECs. Tracking of single labeled cells shows that the vast majority of capillary ECs undergo elongation and interestingly, participate in luminal migration during this developmental period. Furthermore, we find that regression events are not mediated by apoptosis but rather the coordinated repositioning and integration of ECs into existing vessels. Intriguingly, the rate of EC migration gradually decreases and eventually arrests in adulthood with adult ECs displaying positional stability even over a period of 4-weeks. We next sought to understand the mechanisms by which the adult vascular plexus is able to maintain vascular function and integrity during homeostasis. By carrying out targeted laser ablations to inflict localized subcellular damage, we find unexpectedly that ECs do not respond with proliferation but instead rapidly compensate for their neighbors by elongation or migration towards the injury site to mediate vascular repair. Intriguingly, we also observe that injury inflicted on the plasma membrane of individual ECs results in a pinching off and discarding of damaged membrane in what is likely an exocytic mechanism of
plasmalemmal self-repair. This is a phenomenon that has not previously been described in ECs and we are currently investigating the role that caveolae (enriched in ECs) could be playing in the self-repair process by utilizing Caveolin-1 knockout mice. Overall, this study sheds light upon fundamental mechanisms by which ECs maintain and control vascular homeostasis in a live mammal.

P22

**Epithelial cell division opens the door for macrophage tissue invasion in the *Drosophila* embryo**

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Tissue-resident macrophages are indispensable in embryo- and organogenesis, and also influence the progression of major types of tumors in both invertebrates and vertebrates [1,2]. These functions rely on their ability to invade into interfaces formed by tissues, yet how surrounding tissues influence these capabilities in vivo has been the focus of little study. We study such invasive migration in the early *Drosophila* embryo, where macrophages migrate along the inner basal side of an epithelia and eventually separate this ectodermal layer from the underlying mesoderm, thus invading between these tissues [3,4]. Ectoderm-mesoderm attachment is mediated by a thin extracellular matrix (ECM). Previous experiments imply that the time for macrophage entry is influenced by the mechanical resistance of the surrounding cells [4]; however, what determines the choice of when macrophages start to invade remained mechanistically unclear. Here we show that breaching of the ectoderm-mesoderm barrier by the first macrophage always correlates with the mitotic rounding or division of the ectodermal cell at the entry site. This correlation holds even when the timing of division is altered genetically or pharmacologically: increasing the division rate fosters invasion, and decreasing division frequency impedes it. If ectodermal divisions are completely blocked, macrophages cannot invade the tissue. It is known from in vitro work that cells gradually lose focal adhesions during mitotic rounding until only weak reticular contacts remain [6]. In our in vivo context, the facilitation of invasion by division appears to act through the dissolution of these same focal adhesions in the basally-dividing ectoderm cells flanking macrophage entry; we observe the disappearance of Vinculin-mCherry-marked ECM attachments facing the mesoderm just prior to macrophage advancement. Loosening the ecto-meso attachment by knocking down focal adhesion components specifically in the ectoderm through RNAi of Vinculin, Talin, or beta-PS Integrin facilitates macrophage invasion. Thus, we show that focal adhesions at the tissue edge prevent cells separation by a migrating cell, and that adhesion loss caused by division is required for initial breaching of this barrier. Our study demonstrates how cell division at a tissue edge influences cell invasion into a confined environment in vivo. These results may also be relevant for immune cell infiltration of solid tumors and cancer cell invasion into confluent tissues. [1] Wood W. et al. *Dev Cell.*;40(3):221-233 (2017)[2] Ovchinnikov D, *Genesis* 46:447-462 (2008)[3] Siekhaus D.E., et. al, *Nat. Cell Biol.*, 12(6), pp. 605-610, (2010)[4] Ratheesh A. et al., *Developmental Cell*, 45, 331-346 (2018)[5] Lock J. et al. *Nat Cell Biol.*;20(11):1290-1302. (2018)

P23

**Syntaxin 4 Regulates Cardiac Conduction in Vertebrate Cardiomyocytes**

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Arrhythmias are the most common cause of morbidity/mortality in patients with congenital heart diseases and primary arrhythmic disorders. However, the etiologies of these congenital arrhythrogenic disorders (CADs) remain poorly understood. We identified a patient with a spectrum of abnormalities, including dilated cardiomyopathy and a failure to respond to transient cardiac pacing, who ultimately required heart transplantation. Whole-exome sequencing of this proband identified a homozygous non-conservative substitution in the SNARE protein Syntaxin 4 (STX4) locus that previously had not been implicated in playing a role human disease or cardiac function. To understand this requirement of Stx4, we generated zebrafish stx4 mutants that, in contrast to the early embryonic lethality of murine knockouts, develop a spectrum of defects reminiscent of the patient’s, including pericardial edema, linearized hearts, and bradycardia. Due to the apparent conservation of this phenotype, we first assessed cardiomyocyte (CM) differentiation, but failed to observe a difference in CM number in stx4 mutants. Closer analysis of the developing heart indicated a requirement for Stx4 in cardiac conduction, as mutant hearts exhibited a slower calcium transient velocity, and for vesicle trafficking, as mutant CMs had fewer docked vesicles vs. WTs. Our data also suggest that the conduction defect may be autonomously required in CMs, as mutant hearts are competent to respond to sympathetic stimulation, despite the absence of autonomic innervation by 72 hours-post-fertilization (when the cardiac phenotype is fully penetrant). Our data obtained by modeling a previously unreported human disease variant in zebrafish demonstrates an unanticipated/conserved requirement for Stx4 in cardiac function. Because the role of SNAREs in cardiovascular disease is not understood, our investigation of Stx4 in normal cardiac function may elucidate novel therapeutic targets for the treatment of CADs.

P24

Endochondral ossification of proximal femur calcified cartilage tissue in Space and on Earth
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Previously we have reported that calcified cartilage tissue in the proximal femur of 19-week male mice flown in microgravity for 30 days during the Bion-M1 spaceflight experiment underwent accelerated secondary endochondral ossification due to spaceflight factors that can include mechanical unloading in microgravity as well as elevated space radiation. In this study we used archival bone tomography data to extend our prior studies to tissues from 16-week female mice flown in microgravity for 37 days in the NASA Rodent Research 1 (RR1) experiment, and also to ground hindlimb unloaded (HU) mice. Specifically, the proximal femur from female mice was imaged using microcomputed tomography with 1 micron resolution on a Bruker 1272 MicroCT. Tomographies were 3D-reconstructed to segment the femoral head volume and respective fractions of calcified cartilage and mineralized bone tissue. Using this approach, we determined that on the RR-1 experiments, using C57BL/6 female mice exposed to microgravity (n=8), 50% of femoral heads showed endochondral ossification versus only 16.7% in ground controls (n=6). In contrast, normal secondary ossification in the proximal femur of female mice is reported to occur much later, at about 25 weeks. These findings strongly suggest that spaceflight in microgravity accelerates secondary ossification of femoral head cartilage, with the premature loss of the epiphyseal plate and its chondroprogenitor stem cell populations required for the final stages of long bone growth. Since spaceflight in microgravity includes exposure to both mechanical unloading and moderately elevated levels of space radiation, we sought to determine if rapid endochondral ossification
was also inducible in HU mice on earth, subjected to mechanical unloading, but not space radiation. Female B6129SF2/J mice at 16 weeks of age were subjected to 30 days of HU, and while trabecular bone loss was readily observed, quantification of calcified cartilage and bone in the femoral head showed no differences in ossification with low levels occurring similarly in both groups (7.7% in HU, with n=13, and 20% in normally loaded, with n=10). These results suggest that accelerated endochondral ossification of proximal femur calcified cartilage and epiphyseal plate closure, both observed in spaceflown male and female mice, may be due to factors other than only mechanical unloading, and could be induced by elevated levels of space radiation and associated oxidative stress, possibly acting in combination with mechanical unloading.

P25

Restoration of cholesterol dependent craniofacial abnormalities by WNT signaling activation in zebrafish
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Mutation of the cholesterol synthesis pathway (CSP) causes an inborn error of cholesterol metabolism which are associated with craniofacial abnormalities. We have previously established that zebrafish (Vu57 allele) harboring mutation of the *hmgcs1* gene, which encodes the first enzyme in the CSP, is associated with craniofacial defects that result from abnormal differentiation of neural crest cells (NCC), multipotent progenitor cells that produce craniofacial structures. However, the molecular process by which the products of the CSP disrupt NCC differentiation remain poorly understood. However, previous studies have shown that cholesterol regulates activation of WNT signaling, which is essential for craniofacial development. We hypothesized that defects in cholesterol synthesis cause defective WNT signaling and consequently disrupt craniofacial development. We performed a combination of pharmaceutical inhibition, gene expression assays, and targeted rescue experiments to test this hypothesis. Our analysis revealed reduced expression of four WNT downstream target genes in the homozygous carriers of the Vu57 allele and reduced *axin2* expression, a validated WNT target gene, in larvae treated with Ro-48-8071, a drug that inhibits the synthesis of cholesterol. Moreover, activation of WNT signaling via treatment with WNT agonist-I completely restored the craniofacial defects present in a subset of animals carrying the Vu57 allele. Collectively, these data suggest that cholesterol is required for WNT signaling and craniofacial development.

P26

Ablating sonic hedgehog regulator suppressor of fused primes cells towards a glial cell fate
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Background: Early neural cell fates are determined by ventral-dorsal activation of the Sonic Hedgehog (SHH) signaling in the neural tube. Spatial activation of SHH gives rise to motor neurons and interneuron precursors, where temporal activation subsequently gives rise to glial cells. Without SHH ligand, negative regulators of the pathway Patched and Suppressor of Fused (SUFU) promote the conversion of Gli proteins into truncated transcriptional repressors. The SHH ligand inhibits the Patched receptor, causing downstream inhibition of SUFU and subsequent activation of full-length Gli proteins. P19
embryonal carcinoma cells are a robust model for investigating neural differentiation. Treatment with retinoic acid (RA) induces neural lineages where neurons form after 10 days and astrocytes form after 17 days in culture. **Hypothesis:** If P19 cell differentiation recapitulates the timing of SHH signaling in the developing neural tube, then altering SHH signaling, through genetic ablation of Sufu, will alter neural fate specification. **Results:** SHH signaling was active 1 day after RA induction and was subsequently re-activated at days 14 and 17 of differentiation. Pathway activation coincided with neural precursor and glial cell differentiation, respectively. Genetic ablation of Sufu induced the expression of SHH target genes Gli1, Ptch1 and Ascl1 in undifferentiated cell cultures. Neuron marker β-III-tubulin signal was attenuated on days 14 and 17 of RA induced differentiation in Sufu−/− cells. This attenuation was accompanied by the appearance of astrocyte marker GFAP at day 14 and an increase in GFAP signal at day 17 with RA induction. Sufu−/− cells also showed GFAP signal on day 17 of differentiation without RA treatment. **Conclusion:** P19 cells mimic the temporal activation of SHH observed in vivo, where overactivation of the pathway through the ablation of Sufu alters the trajectory of differentiating cells towards a glial cell fate.

P27

**Immunological chemokine fractalkine mediates oligodendrocyte formation from postnatal neural precursor cells**

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**Introduction:** Oligodendrocytes produce myelin, an essential component of the central nervous system. In the adult brain, oligodendrocytes are generated via a 2-step process: neural precursor cells (NPCs) in the subventricular zone (SVZ) niche are committed to oligodendrocyte precursor cells (OPCs), which then differentiate into oligodendrocytes. Formation of oligodendrocytes from their multipotent progenitors is regulated by signals present in the NPC niche. The chemokine fractalkine (FKN), which is secreted by neurons in the NPC niche and signals via its sole receptor (CX3CR1), increases oligodendrocyte formation from embryonic cortical NPCs (Voronova et al., Neuron, 2017). CX3CR1 is also expressed in postnatal and adult NPCs and OPCs (reviewed in Watson et al., Neurosci Lett 2020). However, the role of FKN signalling in postnatal NPCs and OPCs is not currently known. **Methods:** We utilized RNA scope to detect Cx3cr1 mRNA expression in postnatal brain. We also injected FKN directly conjugated to fluorophore Alexa-647 (CX3CL1-647) into lateral ventricle of adult brain to identify which cell types bind FKN in vivo. We then infused FKN into the lateral ventricle of adult NPC lineage tracing mice (NestinCreERT2;RosaYFPSTOP/+ ) to assess the role of FKN signaling in de novo oligodendrocyte genesis. To test the direct effect of FKN on precursors, we generated NPCs or OPCs from murine postnatal SVZ neural stem cell neurospheres, in which the only cells that propagate are precursors. Microglia-free NPC or OPC cultures were incubated in the presence of soluble FKN function blocking antibodies raised against FKN or CX3CR1 and analyzed for differences in proliferation, differentiation and survival. **Results:** Here we provide evidence that in addition to microglia, postnatal SVZ NPCs express Cx3cr1 and bind FKN in vitro and in vivo. When FKN is added to microglia-free NPC cultures, it enhances their differentiation into OPCs and oligodendrocytes without affecting precursor proliferation or survival. Inhibition of FKN signalling in microglia-free OPC cultures with function-blocking antibodies inhibits oligodendrocyte differentiation. Finally, infusion of FKN into lateral ventricle of adult NPC lineage tracing mice enhances OPC and oligodendrocyte genesis from SVZ NPCs in vivo. **Conclusions:** In summary, we demonstrate FKN...
signalling is necessary and sufficient for oligodendrocyte genesis from precursor cells. Our results raise the possibility that immunological chemokines, such as FKN, may play an important role in neural precursor function and can be used for engagement of NPCs for enhanced oligodendrocyte production.

P28

The conserved histone deacetylase, HDA-1, functions in cell cycle-dependent and independent roles to promote invasive differentiation


Cell invasion occurs naturally during development; however, it also contributes to tumor progression. Although understudied, data from different cancer subtypes suggests that a dichotomy exists between invasive and proliferative behavior. Our lab uses the C. elegans anchor cell (AC) to study this proliferative-invasive switch during uterine-vulval development. Post-embryonically, the AC invades into the vulval epithelium to form the mature vulva. We have shown that the AC exists in G0/G1-cell cycle arrest during invasion, which requires the nuclear hormone transcription factor, nhr-67 (tailless/Tlx). Loss of nhr-67 results in mitotic, non-invasive ACs. nhr-67 maintains AC arrest by regulating the expression of the cyclin-dependent kinase inhibitor, cki-1 (p21/p27). Induced expression of CKI-1 is sufficient to restore invasion in an nhr-67-depleted background, suggesting that the G1/G0 state is required for invasive activity. Differentiation of invasive behavior is also regulated by chromatin modifiers that act downstream and/or parallel to G0/G1 arrest. We have found that the histone deacetylase, hda-1, promotes AC invasion by positively regulating pro-invasive gene expression; however, whether hda-1 regulates invasion by controlling cell cycle arrest is unknown. We and others have recently shown that key pro-invasive transcription factors, nhr-67/Tlx, hlh-2/E, fos-1a/Fos, and egl-43/Evi1, maintain the AC in a post-mitotic, pro-invasive state by acting as part of gene regulatory network composed of two subcircuits. nhr-67, egl-43, and hlh-2 function in a type 1 coherent loop with positive feedback to maintain the AC in a post-mitotic state and facilitate invasion, while fos-1 acts in a cell cycle-independent subcircuit to promote invasion. Using genetic approaches, CRISPR-Cas9 genome engineering and high-resolution imaging, we explored the regulatory relationships between HDA-1 and these key transcription factors. We find that hda-1 functions in the AC to maintain G1/G0 arrest, and that loss and depletion of hda-1 results in mitotic, non-invasive ACs. We show that in the AC, hda-1 regulates the activity of NHR-67, FOS-1a, and HLH-2. Interestingly, induced expression of CKI-1 fails to restore invasion in hda-1-deficient ACs, but is able to rescue the mitotic defect of hda-1-depleted animals. These results suggest that hda-1 functions in both cell cycle-dependent and independent subcircuits to maintain the post-mitotic, pro-invasive state of the AC.
**Centrosome Assembly and Functions 1**

**P29**

**Asymmetrical maturation of de novo assembled twin centrioles**

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Centrioles organize centrosomes and cilia, performing multiple functions such as signaling, cytoskeleton remodeling and cell motility. Centrioles are present in all eukaryotic branches, being ultrastructurally conserved but diverse in function and biogenesis. While in some species centriole duplication is coupled with the cell cycle, *de novo* centriole biogenesis is known to occur in several cells/organisms that lack pre-existing centrioles (such as plants). However, besides the conservation of several proteins and the structures themselves, little is known regarding the mechanisms that regulate *de novo* biogenesis of these organelles. Plant vegetative cells are devoid of motility, however the sperm cells of several plant species are flagellated. Therefore, centrioles arise *de novo* during spermatogenesis in early land plants with motile sperm, providing an excellent system to investigate centriole evolution and assembly. We are studying the pathways underlying centriole and locomotory apparatus assembly during spermatogenesis in the moss *Physcomitrella patens*. Our ultrastructural analyses allowed us to recognize distinct morphological stages and structures assembled, with both conserved and unique features being identified in developing sperm cells. Furthermore, 3D electron tomography has allowed us to characterize the bicentriole structure - a unique structure from which two longitudinally oriented centrioles assemble *de novo*, connected by their cartwheel. Additionally, our electron tomography data also revealed that during cell maturation the two sister centrioles become asymmetrical, both at cartwheel and microtubule size. Consistently with a conservation of structures and function, some core centriolar genes are known to be conserved across ciliated species (e.g. SAS-6, BLD10/CEP135 and POC1). Our data suggests that such centriolar components have conserved their functions in cartwheel (SAS-6 and BLD10/CEP135) and centriole (POC1) assembly across evolution. Moreover, SAS-6 and POC1 localizations highlight the cartwheel elongation observed in these cells and support the asymmetry between twin centrioles. This work has allowed us to shed light into the pathways for *de novo* centriole assembly and its evolution. Moreover, our data also reveals particularities of *Physcomitrella patens* cartwheel and centriolar length, as well as their asymmetrical maturation. The regulation and functional consequences of such asymmetry are currently being explored.

**P30**

**Condensation of pericentrin proteins in human cells illuminates phase separation in centrosome assembly**

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The centrosome is a membraneless organelle that serves as the main microtubule organizer in animal cells. It orchestrates diverse cellular processes such as cell signaling, polarization, and division. At the onset of mitosis, centrosomes concomitantly expand their pericentriolar material (PCM) and microtubule-nucleating activities to ensure faithful cell division by acting as mitotic spindle poles. The PCM is a complex ensemble of proteins enriched with coiled-coil domains and low-complexity regions.
How PCM proteins are recruited and held together in the absence of a lipid membrane to form a micron-sized organelle remains elusive. Here we found that endogenously GFP-tagged pericentrin (PCNT), a conserved PCM scaffold protein important for centrosome organization, condenses into dynamic granules specifically during mitotic entry in cultured human cells. Furthermore, the N-terminal segment of PCNT, enriched with conserved coiled-coil and low-complexity sequences, undergoes concentration-dependent condensation. Formation of PCNT “condensates” exhibits characteristics of liquid-liquid phase separation, including a sharp phase transition at a concentration threshold, as well as coalescence, deformability, and rapid fluorescence recovery after photobleaching the condensates. We further found that these PCNT condensates selectively recruit endogenous PCM components and nucleate microtubules in cells. We propose that coiled-coil and low-complexity sequences, two prevalent sequence features in the centrosomal proteome, are preserved under evolutionary pressure—at least in part—to drive phase separation, a process that bestows upon the centrosome a distinct material property critical for its assembly and functions.

P31

The kinase ZYG-1 mediates client protein selection to drive centriole duplication

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Centrioles are microtubule-based cylindrical structures, that are characterized by a nine-fold radial symmetric arrangement of microtubules around a central “cartwheel-like” structure. They aid in establishing spindle poles and serve as basal bodies of cilia. Centriole duplication is under tight control and happens only once each cell cycle. Mis-regulation of the process has been linked to human diseases such as cancer and primary microcephaly. The centriole proteins - ser/thr kinase ZYG-1, and coiled coil proteins SAS-7, SPD-2, SAS-5, SAS-6 & SAS-4 were identified in C. elegans and their homologs have since been found across genera. The hierarchical requirement of these proteins in centriole biogenesis is well conserved with only minor variations. Although a general theme of centriole assembly has been worked out, mechanistic details of the process such as the critical substrates of the master regulator ZYG-1 and the importance of substrate phosphorylation are still not completely understood. An inability to obtain sufficient quantities of full-length recombinant centriole proteins has hindered studies to address these questions. This is due to their accumulation in the insoluble fraction when expressed in heterologous systems such as E. coli. Taking an alternative approach, we purified ZYG-1, SAS-5, SAS-6 and SAS-4 from inclusion bodies and successfully refolded them in vitro. Our biophysical and biochemical analysis indicate that they have attained their native conformation. Using these proteins, we demonstrate a novel direct interaction between ZYG-1 and SAS-5 and showed that SAS-5 is a ZYG-1 substrate in vitro. Using mass spec, we identified phosphorylated serine and threonine residues within SAS-5 and found that many of these were conserved among nematodes. Several of these sites were located within a large conserved motif in the N-terminus that we found could mediate interaction with either ZYG-1 or SAS-4. Strikingly, the precise pattern of phosphorylation within this motif dictates 1) which binding partner is able to dock, and 2) the ability of ZYG-1 to phosphorylate additional sites in SAS-5. To validate our in vitro results we used CRISPR to modify some of these residues in the endogenous sas-5 gene and found sporadic centriole duplication defects and temperature-dependent lethality. Our results suggest that during centriole assembly, ZYG-1-mediated phosphorylation of SAS-5 within this motif is a key event that helps drive the incorporation of SAS-4 into the growing centriole.
Unconventional centrosome organization in malaria parasite

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Malaria still kills about 400,000 children every year. Pathogenesis occurs during replication of the *Plasmodium* parasite in human red blood cells. Rapid proliferation is a key strategy for parasite survival and ensures efficient transmission to the mosquito vector. The mechanisms underlying its atypical cell division mode are, however, completely understudied. Key specificities are the absence of cell cycle checkpoints and asynchronous divisions. Only after multiple rounds of closed mitosis the nuclei are packaged into budding daughter cells. Parasite centrosomes are acentriolar and have an unconventional organization that is neither similar to fungal spindle pole bodies nor vertebrate centrosomes. Using correlative light-electron microscopy and STED nanoscopy we aim to generate a first working model describing this atypical microtubule organizing center. We, thereby, uncovered a multipartite structure with centrosomal components outside and inside the nucleus. A novel intranuclear compartment, which is devoid of chromatin, houses distinct microtubule nucleation sites. Centrosomal microtubules undergo three distinct organizational states prior to chromosome segregation. After accumulating tubulin in intranuclear foci the parasites polymerize hemispindles with branches sometimes reaching beyond the nuclear boundary. Preliminary data indicates that non-polymerized tubulin accumulations are specific to nuclei actively replicating their DNA. Only thereafter, centrosomes duplicate and form highly compact mitotic spindles. Using this organizational framework we can start investigating how cycles of replication and chromosome segregation are orchestrated at the level of individual nuclei. Those findings broaden our scope of centrosome biology beyond what has been studied in model organisms and will advance our understanding of how this parasite rapidly and effectively establishes infection.

Assessing the role of SAS-7 in the assembly and mitotic expansion of the centrosome

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Centrosomes are the major microtubule organizing centers (MTOCs) of animal cells that facilitate spindle assembly and chromosome segregation. Understanding the mechanisms of centrosome assembly is important for cancer therapeutics, as centrosome aberrations lead to chromosome instability and are often observed in tumor cells. Centrosomes consist of a centriolar core that organizes an extended proteinaceous matrix called the pericentriolar material (PCM). The PCM docks γ-tubulin containing complexes that nucleate microtubules. To accommodate the increased demand for microtubules during spindle assembly, the PCM matrix expands 5 to 10-fold during mitotic entry in a process called centrosome maturation that is controlled by polo-like kinase 1 (PLK1). To understand how centrioles direct the assembly of the mitotic PCM, we are using the *C. elegans* embryo as a model system. In *C. elegans*, PLK1 localizes to centrioles by binding to an essential docking site on SPD-2 (homolog of human CEP192). In the absence of SPD-2 or its PLK1 docking site, centrioles remain nearly naked, with only a thin layer of PCM around them. The question then, is how centrioles recruit SPD-2.
Prior work identified a centriolar protein called SAS-7, which is thought to localize to the outer centriole wall. SAS-7 was shown to contain two SPD-2 binding sites at its C-terminus, suggesting it plays a role in SPD-2 incorporation to the centrosome (Sugioka et al., eLife 2017;6:e20353 DOI:10.7554/eLife.20353). SAS-7 has been difficult to deplete by RNAi and therefore its role has been characterized primarily using a hypomorphic temperature sensitive mutant that deletes the most C-terminal SPD-2 binding site and partially destabilizes the protein. Although this mutant compromised PCM organization to some extent, the PCM still expanded during mitosis relatively normally. We have developed a new RNAi-based means to assess the SAS-7 loss-of-function phenotype. We are currently using this method to examine the effect of removing SAS-7 on the ability of centrioles to assemble PCM during interphase and expand PCM during mitotic entry, and will present these results at the meeting.

**P34**

**The splicing factor SON is required for the expression of centrosome factors and the cytoskeletal organization required for centriole assembly**

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As the primary microtubule organizing center of dividing cells, control of the number of centrosomes is critical for cell division, trafficking and cilium formation. Regulation of centrosome number occurs through the precise assembly of centrioles that reside in the center of centrosomes. There are multiple mechanisms of centriole assembly control including at the transcriptional level. To examine transcriptional regulation more closely, we utilized a technique that isolates centriole assembly from other cell cycle processes. We tested five splicing factors originally identified as required for centriole assembly (Balestra et al, 2013), and found that amongst these only SON is specifically required for this process. Although centriole assembly is abolished when SON is reduced, early centriole assembly events occur. Whole genome mRNA sequencing identified thousands of genes whose expression and splicing is affected by the reduction of SON, with an enrichment of genes involved in the microtubule cytoskeleton. By focusing on proteins associated with centrosomes and centriolar satellites we identified CEP131 and CNTROB (centrobin) as candidates involved in centriole assembly. SON is required for the proper splicing and expression of both of these genes, although reduction of either protein on its own, or in combination, has a less severe centriole assembly defect than does reduction of SON. Using fluorescence microscopy and electron tomography, we observed key differences in the cytoskeletal network around the centrosomes that likely contribute to centriole assembly defects. This establishes that SON is required for centriole assembly, partially through its activity in splicing CEP131 and CNTROB as well as through control of the cytoskeletal network around the centrosome.

**P35**

**Phosphorylation of the half-bridge protein Sfi1 regulates spindle pole body maturation**

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The spindle pole body (SPB), the yeast equivalent of the mammalian centrosome, is crucial for mitotic spindle formation, chromosome segregation, and nuclear positioning. Proper bipolar spindle formation
requires duplication of the mother SPB in an ordered, tightly regulated series of steps, the details of which remain unclear. Understanding how SPB duplication occurs requires a method of analyzing protein-protein interactions that take place at both the mother SPB, as well as the daughter SPB throughout its stages of maturation. We created a novel method combining structured illumination microscopy (SIM) with acceptor photobleaching Förster resonance energy transfer (FRET), hereafter referred to as SIM-FRET. This approach allowed us to examine assembly of the new SPB. To our surprise, this structure is not an exact replica of the mother SPB, suggesting that protein interactions at the developing SPB are dynamic and change during the duplication process. SIM-FRET showed positive interactions between bridge component Sfi1 and the SPB core proteins Spc42 and Spc29 dependent on phosphorylation; interactions that were also confirmed by immunoprecipitation and two-hybrid experiments. Based on our data, we propose that phosphorylation regulates a switch between Sfi1 binding to Spc42 and Spc29 that is crucial for the highly ordered process of SPB duplication and assembly.

P36

A centrosomal Polo and Spd-2 oscillation regulates mitotic centrosome size in early Drosophila embryos

Centrosomes help to organise the two poles of the mitotic spindle and are formed when centrioles recruit pericentriolar material (PCM) around themselves. Centrioles generally organise very little PCM in interphase, but the PCM expands dramatically as cells prepare to enter mitosis. How mitotic centrosome size is determined, and how the two spatially separated centrosomes in a cell grow to the same size, is mysterious. A putative pathway of mitotic centrosome assembly has been elucidated in Drosophila. The centriole-associated protein Spd-2 recruits Polo kinase and Cnn (Cep192, PLK1 and Cdk5Rap2, respectively, in humans) to the surface of the mother centriole. Polo phosphorylates Cnn, initiating the assembly of a Spd-2/Polo/Cnn scaffold that then expands outwards around the mother centriole. This scaffold recruits other PCM clients, such as the γ-TuRC, to the mitotic centrosome. Here, we develop methods to quantitatively describe the kinetics of mitotic centrosome growth in the early Drosophila embryo. We find that the mitotic centrosomes grow to a relatively constant size during successive rounds of division, even as their numbers increase exponentially. Unexpectedly, centrosomal levels of Polo and Spd-2 oscillate during each division cycle, with levels peaking and then declining prior to mitotic entry. The Plk1/Spd-2 oscillation is adaptive: when the cell cycle is short centrosomes recruit Plk1/Spd-2 quickly but for a short time and the centrosomes grow quickly, but for a short time; when the cell cycle is longer, centrosomes recruit Plk1/Spd-2 slowly but for a longer time, and the centrosomes grow slowly, but for a longer time. We speculate that this adaptive centrosomal Plk1/Spd-2 oscillation allows centrosomes to reciprocally regulate their growth rate and growth period to maintain a relatively constant size during early embryonic development.
Chromatin, Chromosomes, and Nuclear Organization

P37

Regulation of sumoylation is key to condensate formation and telomere clustering in ALT cancer
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Telomerase-free cancer cells rely on an alternative lengthening of telomeres (ALT) pathway that employs homology-directed DNA repair to maintain telomere length for immortality. One hallmark of ALT cancer is the re-localization of promyelocytic leukemia (PML) nuclear bodies to telomeres to form APBs (ALT-associated PML bodies) and clustering of telomeres in APBs to provide repair templates for telomere elongation. However, the mechanisms for APB assembly and telomere clustering are unknown. We observe that APBs exhibit liquid properties, leading us to hypothesize that APBs condense as liquid droplets to cluster telomeres via coalescence. Furthermore, since DNA damage on telomeres induces APB formation, sumoylation of telomere-binding proteins is important for APB formation, and interactions between SUMO (small ubiquitin-like modifier) and SIM (SUMO interaction motif) can drive liquid condensation, we hypothesized that telomere protein sumoylation, as a result of DNA damage response on shortened/stressed telomere, nucleates APB condensates through SUMO-SIM interactions. To test our hypothesis, we developed an optogenetic approach using a photocaged chemical inducer of protein dimerization. We found that recruiting SUMO or SIM directly to telomeres with dimerization, without DNA damage, induced de novo APB assembly by liquid-liquid phase separation and drove telomere clustering via droplet coalescence in ALT cells. Moreover, such condensation and clustering were also observed in telomerase-positive non-ALT cancer cells that do not sumoylate telomeres or form APBs following DNA damage. Meanwhile, recruiting SUMO ligase to telomeres led to telomere clustering in ALT cells but not in non-ALT cells. These findings suggest that activation of sumoylation is a critical step in enabling APB condensation and telomere clustering in the ALT pathway.

P38

Building a nuclear state landscape in human iPSCs: integrated analysis of the organization and function of nuclear bodies
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The Allen Institute for Cell Science is developing a state space of stem cell structural signatures to understand the principles by which cells reorganize as they traverse the cell cycle and differentiate. To do this, we have developed a pipeline that generates high-replicate, dynamic image data of cell organization and activities in human induced pluripotent stem cell (hiPSC) lines (the Allen Cell Collection at www.allencell.org). To study the organization of the nucleus at multiple spatial scales, 17 of these lines express an endogenous monoallelic EGFP-tagged protein that represents a particular key nuclear structure. Tagged structures include major nuclear landmarks (nuclear lamina, pores, speckles and nucleoli), chromatin structure (histone H2B, HP1-beta, and EZH2), key proteins in chromatin looping (CTCF, SMC1A), chromatin loci (telomeres and DNA replication sites), RNA polymerase, and two pluripotency transcription factors. We take advantage of the thousands of 3D single cell images collected for each structure and use our image analysis tools combined with deep learning-based label free technology to create integrated models and analyses of three key nuclear reference structures: the
lamina, nucleoli and speckles. We find that the total number of speckles scales with nuclear volume, suggesting that new speckles are created, either de novo or by fission, as cells progress through interphase. We also find a scaling relationship between the size of nucleoli and the size of speckles that is independent of each of their scaling with nuclear size, indicating a mechanism by which speckles and nucleoli coordinate their total size. Further, in collaboration with the 4D Nucleome, these integrated models are being expanded to incorporate models of the 3D genome that are computationally generated by combining image-based spatial constraints with 3D genome mapping assays. We are also investigating the functional role of these key nuclear structures with an initial focus on the nucleolus. We performed a CRISPRi pooled screen using a cell line with 2 nucleolar markers tagged with fluorescent proteins as well as the CRISPRi machinery. We screened for genes that perturbed nucleolar morphology as measured by a flow based assay and are in the process of validating the hits by live cell imaging. The primary hits from the screen include genes that regulate both transcription and epigenetics, preliminarily hinting at a relationship between nucleolar structure and regulation of gene expression in hiPSCs. In summary, we presented new integrated models of 4D nucleome organization in hiPSCs along with analysis and a functional screen that together suggest new regulatory connections between nuclear bodies as well as new insights into the connection between their morphology and function.

P39

New potential role of the PI3KC3 in the nucleolus
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The nucleolus is a nuclear compartment where ribosomal RNA (rRNA) is being synthesized, processed, and assembled with ribosomal proteins to form ribosomes. The sequential stages of ribosome biogenesis are reflected in the architecture of the nucleolus comprising three sub-compartments: fibrillar center (FC), dense fibrillar component (DFC) and granular component (GC). Phosphoinositides are small membrane phospholipids implicated in organelle trafficking and cellular signaling. Phosphatidylinositol 3-monophosphate (PI3P) is a key component in the vesicular trafficking, as well as autophagy and mTOR signaling. Although PI3P mainly localizes on the endolysosomal system organelles, its localization was also observed within the nucleolus (Gillooly et al, 2000 EMBO J). In contrast, phosphatidylinositol 3-kinase catalytic subunit type 3 (PI3KC3), which is considered the main cellular source of PI3P, has never been investigated in the nucleus before. In the present study, we demonstrate that PI3KC3 is present in the nucleus of BALB3T3 cells, as shown by immunofluorescence and cell fractionation experiments. Further analysis by confocal microscopy revealed that PI3KC3 is confined to the FC region where it colocalizes with upstream binding factor (UBF), a transcription factor required for expression of rRNA. In addition, pull-down of GFP-UBF co-immunoprecipitated PI3KC3 from BALB3T3 cells. Furthermore, inhibition of RNA polymerase I (Pol I) with actinomycin D abolished PI3KC3 nucleolar localization, and PI3KC3 binding to UBF. All together, these data indicate that PI3KC3 might play an important, still undescribed, role in the nucleolar organization and function. Additional studies are underway for a better understanding of these events. Acknowledgement: this work is supported by American Society of Hematology Global Research Award and University of Rijeka support grant no. 18-188-1343. I.B. is supported by Croatian Science Foundation (HRZZ-09-2016).
The C-terminal domain of rat DNA topoisomerase IIα is involved in the regulation of catalytic activity and nucleolar localization

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Type II DNA topoisomerase (topo II) is an essential enzyme for resolution of topological problems arising in DNA metabolic processes such as transcription and replication. The enzymes form a heart-shaped homodimer, giving rise to a structure that consist of three domains: N-terminal ATPase domain, DNA cleavage/catalytic core domain, and C-terminal domain (CTD). In vertebrates, two isozymes (α and β) are present. Recently, the C-terminal regulatory domain (CRD), which regulates subnuclear localization and catalytic activity by associating with RNA, was identified in the CTD of rat topo IIβ. In contrast, it is unclear whether a β CRD-like domain is present in the CTD of topo IIα. In this study, we aimed to identify a RNA-mediated regulatory domain in the rat topo IIα CTD. First, to identify the domain associated with the subnuclear localization of the CTD of rat topo IIα, we transiently expressed EGFP-tagged CTD deletion mutants in HEK293 cells. The data indicated that the 1192-1289 region of rat topo IIα CTD was required for targeting the enzyme to nucleoli. Next, to examine whether the 1192-1289 region is involved in the RNA-mediated regulation of catalytic activity, we performed a relaxation assay in the presence of RNA. The relaxation activities of topo IIα ΔCTD (1-1191) and Δ1192-1289 mutants were not inhibited in the presence of isolated cellular RNA, suggesting that the 1192-1289 region of α CTD is involved in the RNA-mediated regulation of catalytic activity in topo IIα. The results of on-bead assays using ΔCTD indicated that the RNA-mediated inhibition of the relaxation activity was caused by an interaction between α CTD and RNA. These results indicated that the proximal CTD of rat topo IIα (1192-1289 region) is involved in the regulation of catalytic activity by associating with RNA, as well as in the localization to nucleoli in interphase cells.

Transcription and chromatin architecture regulation by nucleoporin proteins

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The nuclear pore complex (NPC) components, nucleoporins (Nups), have been proposed to mediate spatial and temporal organization of chromatin during gene regulation. Nevertheless, we have little understanding on the molecular mechanisms that underlie Nup-mediated chromatin structure and transcription in mammals. Here, we show that Nucleoporin 153 (NUP153) interacts with the chromatin architectural proteins, CTCF and cohesin, and mediates their binding across cis-regulatory elements and TAD boundaries in mouse embryonic stem (ES) cells. NUP153 depletion results in altered CTCF and cohesin occupancy and differential gene expression. This function of NUP153 is most prevalent at the developmental genes that show bivalent chromatin state. To dissect the functional relevance of NUP153-mediated CTCF and cohesin binding during transcriptional activation or silencing, we utilized epidermal growth factor (EGF)-inducible immediate early genes (IEGs). We found that NUP153 binding at the cis-regulatory elements controls CTCF and cohesin binding and subsequent POL II pausing during the transcriptionally silent state. Furthermore, efficient and timely transcription initiation of IEGs relies
on NUP153 and occurs around the nuclear periphery suggesting that NUP153 acts as an activator of IEG transcription. Collectively, these results uncover a key role for NUP153 in chromatin architecture and transcription by mediating CTCF and cohesin binding in mammalian cells. We propose that NUP153 links NPCs to chromatin architecture allowing developmental genes and IEGs that are poised to respond rapidly to developmental cues to be properly modulated.

**Cytoskeleton in Membrane Dynamics, Cortex and Organelle organization**

**P42**

**IRSp53: Linking Membrane Dynamics with Cytoskeletal Contractility as Cells Crawl and Exert Forces on Suspended Fibers**

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The Insulin Receptor tyrosine kinase Substrate protein of 53 kDa (IRSp53) plays a key role as a signal transducer, linking the plasma membrane deformations at the cell boundary to changes in cytoskeletal contractility. While previous studies have demonstrated that the absence of IRSp53 leads to impaired protrusion formation, our understanding of its role in modulating cytoskeletal contractility remains in infancy. Additionally, these studies are primarily conducted on flat, 2D assays which do not fully recapitulate the in vivo fibrous extracellular matrix (ECM). Here, we use the non-electrospinning Spinneret-based Tunable Engineered Parameters (STEP) method to manufacture ECM-mimicking suspended nanofibers that allow us to systematically investigate the role of IRSp53 in modulating both protrusion dynamics and cytoskeletal contractility in U-251 glioma cells migrating on 135 nm and 500 nm diameter fibers. We found that while the maximum protrusion length remained similar between IRSp53 knock-out (KO) and wild-type (WT) cells, the rates of protrusion elongation were significantly higher for KO cells on both fiber diameters. These rates correlated with our recently reported curvature-sensitive 3D coiling (wrapping-around the fiber axis) behavior at the protrusion tip. We found that the KO cells exhibited lower maximum coil width and took a shorter time to reach the maximum coil width compared to its WT counterparts on both diameters considered. Naturally, we inquired if the altered protrusion rates were connected with cell shape and cytoskeletal contractility. We found that the KO cells took significantly longer to spread on the suspended fibers and were more rounded in shape (higher circularity) compared to the WT cells. Using our nanonet force microscopy (NFM) platform which enables measurement of single-cell forces, we found that KO cells exerted lower forces compared to its WT counterparts. Lower forces and high circularity contributed to KO cells exhibiting oscillatory migration mode resulting in overall lower migration speeds. The reduced force exertion also translated to higher nucleus thickness for the KO cells. In summary, this work, for the first time highlights the unique role of IRSp53 protein in linking membrane dynamics with cell shape, migration and cytoskeletal contractility driven nucleus shape on ECM-mimicking suspended nanofiber networks.

**P43**

**The roles of cytoskeletal regulating proteins on the modulation of cortical actin and the impact on neutrophil deformation and transit through model pulmonary capillary constrictions**
Neutrophils are vital for host defense, but they can become dysregulated and cause tissue damage. Neutrophil dysregulation in the pulmonary system can cause acute lung injury, and in the most severe cases, acute respiratory distress syndrome. For these reasons understanding neutrophil trafficking and regulation is critical for developing effective immunomodulatory therapies for these pathologies. In the pulmonary tissues, neutrophil trafficking is unique due to the diameter of the capillaries (5μm) being smaller than the diameter of the neutrophils (8μm). This size disparity results in the neutrophil having to deform to pass through the pulmonary capillaries. Additionally, when neutrophils are exposed to chemical stimuli, the cells increase their F-actin levels and stiffen. This results in even greater neutrophil retention when in a disease state, increasing the potential for host tissue damage. To investigate neutrophil stiffening's biochemistry, we use conditionally immortalized neutrophil progenitors that can be genetically manipulated and then differentiated into bonafide neutrophils in cell culture. To quantify the effects of f-MLP and GM-CSF on the stiffness of nonadherent neutrophils, we use a microfluidic platform to measure the transit times through 5μm constrictions. The dimensions and force parameters were chosen to mimic those of the pulmonary capillary system. To compare the distribution of transit times, we plotted Kaplan-Meier curves and compared the trends by analyzing the resulting hazard ratios. Our preliminary results show that the addition of f-MLP slows the rate of transit with a hazard ratio of 0.7943 [95% CI 0.7135 to 0.8000] compared to the unstimulated control. GM-CSF has a similar impact on the rate with a hazard ratio of 0.8070 [95% CI 0.7042 to 0.9248]). The most dramatic effect is in combining stimuli. Compared to unstimulated neutrophils, the rate decreased, resulting in a hazard ratio of 0.5523 [95% CI 0.4799 to 0.6357], and compared to the f-MLP alone, the hazard ratio is 0.6648 [95% CI 0.5691 to 0.7766]). To explore the mechanism of chemically induced neutrophil stiffening, we first pretreated the neutrophils with cytochalasin D to prevent F-actin polymerization. Comparing WT neutrophils with the pretreated sample under f-MLP stimulation, the cytochalasin D group had a faster transit rate, hazard ratio 1.352 [95% CI 1.157 to 1.581]). Also, we are investigating genetic knockouts that are known to be involved with the cytoskeletal organization. Early results show that f-MLP stimulus no longer induces as strong a stiffening response, resulting in faster rates in vinculin⁻/⁻ neutrophils; (“Hazard Ratio 1.109) and in HS1⁻/⁻ (hematopoietic lineage cell-specific protein 1) neutrophils; (“Hazard Ratio 1.412) when compared to WT.

P44

Mechanical coupling of epithelial cell migration and actomyosin cable contraction in the two-layered epidermis of embryonic zebrafish

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During wound healing for the embryonic zebrafish epidermis, restoration of the epidermal boundary is thought to be governed primarily by two cytoskeletal processes: migration of epithelial cells toward the wound and contraction of an actomyosin cable in cells along the wound margin. Previous studies in Drosophila epithelial tissues and cell culture monolayers discovered wound repair proceeds in distinct phases and requires coordination between both processes in monolayered systems ². However, it
remains unclear exactly how these two processes are mechanically coupled or to what extent they interact during wound healing. In zebrafish embryos, purse string formation and cell migration are spatially separated in the two layers of the epidermis: cable formation in the superficial and cell migration in the basal layer. This separation raises the possibility of pinpointing the distinct mechanical contributions of each process with cell layer-specific gene expression. Cytoskeletal actin is crucial in both cable-formation and cell migration. For this reason, we applied a genetically encoded construct (DeAct-GS1) that directly interferes with the polymerization of F-actin in each layer by sequestering actin monomers in a stoichiometric fashion. We predicted that both purse string closure and cell migration are capable of driving closure and can compensate for one another. Fluorescent microscopy and image analysis reveal that cells in the basal layer expressing DeAct-GS1 are less protrusive and have smaller lamellipodial fronts, and do not polarize towards the wound as much as controls. They also appear to initiate independent migration more slowly than control constructs. Mosaic expression of DeAct-GS1 is not sufficient to inhibit wound closure, suggesting when mosaic inhibition of actin affects one process the other process is capable of compensating in order to facilitate wound closure. Here we compare our cells expressing our DeAct-GS1 construct to DMSO controls and nearby cells devoid of expression.


P45

**Isoform-specific functions of Dystrophin in Drosophila tissue morphogenesis.**

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Dystrophin (Dys) is a spectrin superfamily protein that can link the actin cytoskeleton to the ECM via the transmembrane connector dystroglycan (Dg). However, Dys (and its vertebrate paralog Utrophin) also makes a set of shorter C-terminal Dg-binding isoforms that lack the main, N-terminal actin binding domain. A division of labor among these long "linker" and short "scaffold" isoforms has emerged in the literature, but their developmental roles remain unclear. Here we analyzed a Drosophila Dys allele that prevents expression of "linker" forms, and compared it to three null alleles. All allelic combinations are viable and yield a characteristic defect in wing vein pattern. However, the isoforms diverge in function in oogenesis. The loss of "linker" forms has minimal impact on egg chamber development, while complete loss of Dys leads to multiple defects. Thus, the oogenesis functions of Dys can be attributed to the "scaffold" isoforms. In particular, we find that “scaffold” isoforms rescue the egg elongation defect of Dys, and largely restore F-actin stress fiber orientation. We also show these isoforms play an important role in basement membrane fibril deposition, which contributes to egg shape. Localization studies indicate that there is a complex long-range organization of Dys in the cortex, involving different isoforms and/or different domains of the longer forms. This Dys pattern has a dynamic relationship with cortical F-actin across stages of egg chamber development. Understanding this isoform diversity and structure-function properties in developing tissues has implications for treating muscular dystrophies in which Dys is lost or compromised, but can be partially restored or replaced using genetic manipulations.
Ctdnep1 and Eps8L2 regulate dorsal actin cables for nuclear positioning and cell migration
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Cells actively position their nuclei within the cytoplasm for multiple cellular and physiological functions. Different cell types position their nuclei away from the leading edge during cell migration. In migrating fibroblasts, nuclear positioning is driven by dorsal actin cables connected to the nuclear envelope by the LINC complex on Transmembrane Actin-associated Nuclear (TAN) lines. Dorsal actin cables are required for the formation of TAN lines. However, how dorsal actin cables are organized to promote TAN lines formation is unknown. We have identified a role for Ctdnep1/Dullard, a nuclear envelope phosphatase, and the actin regulator Eps8L2, on nuclear positioning and cell migration. Ctdnep1 and Eps8L2 directly interact, and this interaction is important for nuclear positioning and cell migration. We also found that Ctdnep1 and Eps8L2 are involved in the formation and thickness of dorsal actin cables required for TAN lines engagement during nuclear movement. We propose a novel mechanism to regulate actin at the nuclear envelope for nuclear positioning in migrating fibroblasts.

P47

Jaw1 Is a Novel Protein to Maintain the Golgi Ribbon Structure
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Jaw1/LRMP is a type II membrane protein that is localized to endoplasmic reticulum (ER) and outer nuclear membrane. We previously reported that Jaw1 has a role to maintain nuclear shape as a KASH protein via interaction with SUN proteins, which is an inner nuclear protein, and tubulin and form the linker of nucleoskeleton and cytoskeleton complex. Moreover, our preliminary experiment indicated that Jaw1 depletion also causes the Golgi morphological change, therefore, we focused on a novel function of Jaw1 as a regulatory protein of the Golgi morphology. In this study, we show that Jaw1 has a role to maintain the Golgi morphology, and also show the morphological and functional change in detail. First, we carried out siRNA-mediated Jaw1 knockdown (Jaw1 KD) in B16F10 cells derived from mouse melanoma, and stained GM130, a cis-Golgi marker, to compare the Golgi morphology between the control and Jaw1 KD cells. The result showed that Jaw1 depletion cause Golgi fragmentation. In order to investigate the morphological change in detail, we performed observation by the transmission electron microscopy (TEM) and co-immunostaining with GM130 and golgin97, a trans-Golgi marker. In the TEM image of the Jaw1 KD cells, the Golgi ribbon structure was disorganized. Furthermore, the line plot
profile and 3D reconstruction of co-immunostaining revealed that the fragmented Golgi in Jaw1 KD cells still maintained its cis-trans stacking. Next, to investigate the glycosylation ability in the Golgi fragmented condition, we carried out recombinant DNase1 glycosylation assay, to compare the ratio of immatured DNase1 and matured DNase1. Unexpectedly, the glycosylation ability in Jaw1 KD cells was compatible with the control. Finally, in order to explore the mechanism by which the morphological change occurs and the localization of cytoskeletal components, since the cytoskeleton affect the Golgi cisterna formation via linking Golgi cisterna to microtube network. As a consequence, we found that the abnormal cytoskeletal proteins localization. In summary, we found that Jaw1 KD cells lose the Golgi ribbon structure, which still have intact cis-trans stacking, and have an abnormal cytoskeletal proteins localization. Those data suggest that Jaw1 maintain the Golgi morphology via regulating the cytoskeletal structure.

**P48**

**A method of mounting C. elegans for prolonged fluorescent imaging**

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The hermaphroditic nematode *C. elegans* is a simple yet powerful model organism with a transparent cuticle and short generation time that make it an accessible tool for the study of conserved features across animal phylogeny. Use of time lapse imaging using fluorescent microscopy to study the worm has been limited by the need for an effective method of feeding worms while immobilized for imaging. We adapted a method of mounting worms in a UV-polymerized PEG/acrylate gel solution derived from Burnett and Albrecht [Burnett et al. (2018) Commun Biol. 73(2018). doi: 10.1038/s42003-018-0079-6]. This method fully immobilizes worms while allowing for them to feed during imaging. Worms are unable to release eggs from the uterus, but ovulation proceeds for several hours. This method facilitates ongoing studies of the *C. elegans* oogenic germline, a U shaped syncytium made up of hundreds of cell-like compartments linked to a common cytoplasm. Specimens left in gel for several hours have been observed ovulating embryos at regular intervals. This method will be useful for investigations of oogenesis and germline structure. The time between ovulation events in *C. elegans* worms mounted using this method was tabulated for 12 worms. Across these 12 worms, 25 ovulations were observed. The average time between detachments from the germline was 41.5 minutes, the median time was 37 minutes, and mode was 20 minutes. 68% of detachments in this data set took less than 40 minutes. The method presented here enables long term imaging of the *C. elegans* germline by allowing specimens to feed while fully immobilized. This method presents an opportunity to study the dynamics of cell shape change throughout oogenesis.

**Mechanotransduction and Single Cell Migration**

**P49**

**The Landscape of Interactions Between the Cytoskeleton, Mechanoresponsiveness, and Cell Behavior**

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Cells intimately interact with their environment, exerting and experiencing physical forces. To navigate the spectrum of physical inputs, cells use a feedback system that allows them to sense and adapt to...
these cues. Several key components of the cytoskeletal network respond to mechanical stimuli, i.e. mechanorespond, suggesting key roles in the mechanical feedback system. Non-muscle myosin (NMII), an ATP-driven motor protein, is one of the cytoskeleton’s mechanoresponsive proteins. The functional “myosin II monomer” is a hexamer containing two heavy chains and four light chains. All three paralogs of NMII assemble into multimeric complexes called bipolar thick filaments (BTFs) that function at the cortex. BTF assembly is regulated across all NMIIIs via light chain phosphorylation, while paralog specific control is regulated via heavy chain phosphorylation. Previous work from our lab found that mechanoresponsiveness of one NMII paralog, NMIIB, is dependent on the fraction of basal NMIIB which is assembled at the cortex and that this was tunable by altering heavy chain phosphorylation state. We now ask what the behavioral outputs are for alterations in NMIIB assembly and mechanoresponsiveness. To answer this question, we have exogenously expressed NMIIB heavy chain phosphorylation mutants in a panel of cell lines and are quantifying behaviors such as cell motility and metabolism. These cell behaviors are of interest due to the well-established role of NMIIB in migration and the emerging evidence for feedback between mechanotransduction and metabolism. Thus far, we have observed enhanced random migration in leukocytes upon expression of exogenous NMIIB mutants. Initial results suggest that when NMIIB is highly associated with the cytoskeleton, leukocytes exhibit faster migratory potential, regardless of substrate. However, when less NMIIB is associated with the cytoskeleton, migration is enhanced in a substrate-dependent manner. We hypothesize these behaviors occur through altered migratory modes due to changes in adhesion strength and cortical tension. Additionally, we observe altered metabolic oxidative phosphorylation (OXPHOS) in pancreatic cancer cells. When NMIIB is highly associated with the cytoskeleton, OXPHOS is significantly increased, whereas when the pool of monomeric NMIIB is larger, OXPHOS is decreased in comparison to controls. Collectively, these observations suggest a feedback system between NMIIB assembly state and metabolism, leading to integration of mechanics with metabolism to guide cell behavior.

P50

Membrane Ruffling Allows Cells to Sense and Respond to Changes in Viscosity
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Throughout the body, cells are immersed in extracellular fluids (ECF) that are typically orders of magnitude more viscous than water and normal cell culture medium. Changes in the viscosity of biological fluids are associated with physiological processes and diseases ranging from aging to wound healing to cancer. However, unlike the material properties of the extracellular matrix, the effects of viscous fluids on cell behavior have not been thoroughly studied. Treating cells with synthetic viscous ECF, we find that single cells in 2D exhibit a dramatic response to viscous fluid, doubling in spread area, migrating nearly two-fold faster, and generating significantly stronger traction forces. This behavior is conserved across several cell types tested, including NIH 3T3, MDA-MB-231, and HEK293. Our characterization of this response to viscosity leads us to propose a mechanism by which membrane ruffling acts as a sensor of viscosity. We simulated elevated ECF viscosity by adding methylcellulose, an inert thickening agent, to culture medium to increase the viscosity up to a maximum of 11,000 times that of water at low shear. Immediately upon addition of viscous medium, membrane ruffling at the cell’s edge is curtailed and cell spread area begins to expand. We observed that the spread area plateaus at around 200% of its original size after roughly 30 minutes. This behavior is viscosity-dependent, with more viscous solutions leading to greater suppression of membrane ruffling and larger increases in
spreading area. Counterintuitively, single cells migrate nearly two times faster in very viscous medium, and this speed increase persists for at least 24 hours. The strong correlation between viscosity-dependent spreading expansion and cell speed is reminiscent of a previously proposed model, in which strong cell-ECM adhesion promotes cell spreading and, subsequently, motility. Disrupting integrin engagement to ECM or actin polymerization via drug inhibition profoundly perturbs the increase in cell motility and spreading in response to viscous fluid. Therefore, we deduce that the resistance imposed by viscous ECF suppresses membrane ruffling, facilitating greater integrin engagement with ECM and more efficient actin-driven leading edge protrusion. Ultimately, this allows the cell to generate stronger forces and migrate faster. The implications of these findings are two-fold. First, all reported values indicate ECF is much more viscous than that of cell culture medium, and our study suggests the effects of viscosity should be factored in when studying cellular behaviors. Second, understanding the role of ECF viscosity in various diseases could inspire treatments that modulate local viscosity to affect cell behavior.

P51

**Role of actin-binding proteins in sensing mechanical stimulation**

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Molecular mechanisms by which cells sense and directionally migrate in response to mechanical perturbation are not well understood. *Dictyostelium discoideum* cells exposed to a mechanical stimulus show rapid and transient activation of multiple components of the signal transduction network, a response that requires an intact actin cytoskeleton of the cell. However, exactly what aspect of the actin cytoskeleton network is responsible for sensing and/or transmitting the signal is unclear. In this study, we investigated the role of the actin-binding proteins filamin and α-actinin by analyzing shear flow-stimulated responses in cells with or without these proteins. Both of these actin crosslinking proteins showed rapid and transient relocation from the cytosol to the cortex following 2 sec stimulation with shear flow. To detect activation of the signal transduction network activation in the presence or absence of these actin-binding proteins, we used fluorescently-tagged Ras binding domain biosensor that detects active Ras and was previously shown to relocalize to the cortex following mechanical stimulation. Ras activation following stimulation with shear flow was significantly more robust in filamin-null cells expressing full-length filamin compared to cells expressing empty vector. This effect was not due to altered adherence of cells to the substrate. Reduced responsiveness appears to be specific to mechanical stimuli since response to global stimulation with a chemoattractant was comparable between cells with or without filamin. In contrast, response to acute mechanical stimulation was similar in α-actinin-null expressing α-actinin or empty vector. These results suggest that filamin, but not α-actinin, is likely involved in sensing and/or transmitting the mechanical stimulus, and future studies will focus on determining the molecular mechanism of filamin action in this context.

P52

**Sensing the force from within: the nucleus as a mechanosensitive controller of adaptive dynamics**

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Single cells experience physical constrains and various mechanical forces in their 3D tissue environment such as in crowded tissues or during migration in tight spaces. As a consequence, their shape changes and undergoes variable deformations. To ensure tissue integrity and homeostasis during development and in the adult organism, cells need to be able to decode these dynamic shape changes and respond to them accordingly. Mechanical cell deformation in confinement can transform various cell types into a highly contractile and motile amoeboid phenotype, termed stable-bleb migration (Ruprecht et al. 2015, Liu et al. 2015). How cells sense shape deformations and translate this information into myosin II activation regulating cellular mechanics and migration plasticity has remained unclear. Here we show that the nucleus is an elastic mechano-gauge that allows cells to measure physical shape deformation (Venturini et al. 2020). We found that inner nuclear membrane unfolding upon nuclear stretch triggers a calcium-dependent mechanosensing pathway that activates cytosolic phospholipase A2 (cPLA2) and metabolite production of AA which regulates myosin II activity. Furthermore, we show that mechanical nucleus stretch combined with intracellular nucleus positioning enables cells to decode different types of shape deformations. Our data support that the nucleus establishes a functional module for cellular proprioception, enabling cells to sense and interpret shape changes and to adapt their dynamic response behaviour to the 3D microenvironment.

Membrane Trafficking: Cytoskeleton

P53

Myosin II isoforms promote internalization of spatially distinct clathrin-independent endocytosis cargoes through modulation of cortical tension downstream of ROCK2

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Although the actomyosin cytoskeleton has been implicated in clathrin-mediated endocytosis, a clear requirement for actomyosin in clathrin-independent endocytosis (CIE) has not been demonstrated. We discovered that the Rho-associated kinase ROCK2 is required for CIE of MHCI and CD59 through promotion of myosin II activity. Myosin IIA promoted internalization of MHCI and myosin IIB drove CD59 uptake in both HeLa and polarized Caco2 intestinal epithelial cells. In Caco2 cells, myosin IIA localized to the basal cortex and apical brush border and mediated MHCI internalization from the basolateral domain, while myosin IIB localized at the basal cortex and apical cell-cell junctions promoted CD59 uptake from the apical membrane. Atomic force microscopy demonstrated that myosin IIB mediated apical epithelial tension in Caco2 cells. Thus, specific cargoes are internalized by ROCK2-mediated activation of myosin II isoforms to mediate spatial regulation of CIE, possibly by modulation of local cortical tension.

P54

Internalization of membrane adhesion proteins during mating in Chlamydomonas occurs through actin and Arp2/3 dependent endocytic mechanisms

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The unicellular, green alga, *Chlamydomonas reinhardtii* has long been used as a model organism for ciliary studies. The cilia are highly conserved in relation to mammalian cilia and are required for mating, among other functions. When mating is induced, membrane proteins, including Sag1, are relocated from a more uniform distribution at the periphery of the cell to the apical and ciliary region where they are required for cell adhesion. This relocation is thought to occur on intracellular microtubules rather than on the surface, which suggests an endocytic mechanism where membrane proteins are internalized on vesicles which are then trafficked throughout the cell. Such a mechanism has never been explored in *Chlamydomonas*. Therefore, we aim to better understand how membrane protein internalization occurs in this organism. For the first time, we show an endocytic mechanism in *Chlamydomonas* dependent on the cytoskeletal proteins actin and Arp2/3, which are also known to be important for endocytosis in other organisms. Further, through comparative genomics we deduced that the mechanism of endocytosis likely to be found in *Chlamydomonas reinhardtii* is clathrin-mediated as the majority of the players important for this mechanism are found in this organism, and the requirement for these players in endocytosis will be investigated. Overall, we have identified an actin and Arp2/3-dependent endocytic mechanism in *Chlamydomonas* essential for steps of ciliary adhesion during mating.

P55

**Spatio-temporal regulation of actin nucleation promoting factors by SH3 domain interactions in yeast endocytosis**

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In yeast clathrin-mediated endocytosis, assembly of a branched actin network drives robust membrane invagination growth that leads to vesicle formation. Actin nucleation promoting factors (NPFs), especially the WASP homolog Las17 and the type-I myosin Myo5, form a complex that regulates actin network growth during endocytosis. SH3 domains are a common feature of nine different NPF interaction partners and thought to mediate binding to the NPF complex, yet the function of these binding events is mostly obscure. In this study, we tested if SH3 domains play a role in the recruitment of SH3 proteins and in NPF regulation. Using live cell fluorescence microscopy, we determined effects of SH3 deletions on assembly of NPFs, actin and the SH3 proteins themselves. Binding competitions between different SH3 domains might dynamically regulate both NPFs as well as SH3 protein recruitment. Indeed, we observed competition dynamics between the SH3 domains of Sla1, a major NPF recruitment factor, and one of the two SH3 domains of Bzz1, a membrane binding protein. The non-competing Bzz1 SH3 domain mediates Bzz1 assembly when the NPF complex is forming while the competing SH3 domain regulates Bzz1 recruitment to endocytic sites later when NPF proteins disassemble. It appears that these SH3 domains act context-specifically in a complex interaction network, potentially providing negative feedback for NPF control. In contrast, deleting the SH3 domain of Abp1, an actin binding protein, did not change either timing or efficiency of Abp1 assembly to endocytic sites. We found slower actin network growth, delay in vesicle scission and most strikingly a split of the NPF complex in *abp1(SH3Δ)* cells. In wild type cells, the key NPF proteins Las17 and Myo5 are located at the invagination base, but in *abp1(SH3Δ)* cells only Myo5 stayed at the invagination base while Las17 located at the invagination tip. Furthermore, we could confirm previous studies showing that coat and actin proteins disassemble much slower off vesicles in *abp1(SH3Δ)* cells. Taken together,
our data suggests that SH3 domains play a role in timing of NPF interactions as well as NPF location within the dynamic endocytic machinery.

P56

**Multivesicular bodies associate with septin-bound microtubules during maturation in the endomembrane system**

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The multi-vesicular body/late endosome (MVB/LE) is a sorting hub that determines cargo fates for either degradation or extracellular expulsion in exosomes. MVB/LEs are dependent on the microtubule (MT) cytoskeleton for movement towards the cell center during their maturation; this movement enhances lysosomal fusion and determines cargo outcomes. The role the cytoskeleton plays in controlling MVB positioning is still poorly understood. Septins (SEPT) are a family of GTPases that assemble into filamentous structures which associate with both the actin/MT cytoskeleton and membranes. Previous work has shown that septins play a role in MVB/LE biogenesis and morphology. Recently, we found that SEPT9 directly interacts with the MVB/LE sorting protein TSG101 and that SEPT2 associates with PI(3,5)P2-rich macropinosome, promoting their fusion to endosomes and lysosomes. Here, we sought to examine the role of septins in the positioning and movement of the MVB/LE. MDCK cells that expressed SEPT9-mCherry at sub-endogenous levels were transfected with the MVB/LE marker CD63-GFP and early endosome (EE) marker EEA1-GFP and imaged live. We found that both EEs and MVB/LEs localized to SEPT9 filaments. Quantification showed that 36% and 51% of EEs and MVBs/LEs associate with SEPT9 filaments, respectively. Interestingly, CD63 compartments that associate with SEPT9 filaments are more likely to remain on SEPT9 throughout the duration of the analysis, whereas EEA1 compartments disassociate more readily. In fixed images of MDCK cells, ~67% of total CD63-GFP compartments localized on septin (SEPT7) filaments, while only ~37% of total EEA1 compartments are present on SEPT7 filaments. Given that MVBs undergo gradual maturation from EEs to LEs, we sought to examine the dynamics of MVBs on septin filaments during different stages of maturation. We found that the majority of EEA1-positive CD63-GFP compartments (~75%) localize on septin (SEPT7) filaments. Similarly, the majority of LBPA (lysobisphosphatidic acid)-negative CD63-GFP compartments (~70%) are present on SEPT7 filaments. Finally, we examined the localization of ESCRT-I (TSG101)-positive compartments and found that 69% of the TSG101-bound microtubules were coated with SEPT7. Collectively, our data reveal that MVBs associate preferentially with septin-bound microtubules, which could be critical for sorting events and MVB maturation and trafficking.

P57

**A septin GDP-activated scaffold of dynein-dynactin motors triggers retrograde lysosome transport**

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Multiple functions of lysosomes including degradation and stress signaling depend on their intracellular motility and positioning. The spatiotemporal distribution of lysosomes is mainly driven by microtubule...
motors, yet the underlying mechanisms of selective motor recruitment and activation are little understood. Here, we have discovered a novel septin GTPase-based mechanism for retrograde lysosome transport. We show that septin 9 (SEPT9) preferentially associates with lysosomes and promotes their perinuclear localization in a Rab7-independent manner. We observed that SEPT9 overexpression enhances lysosome clustering to the perinuclear area, while SEPT9 depletion causes lysosomal dispersal toward the cell periphery. Using inducible peroxisome and mitochondria trafficking assays we found that ectopic targeting of SEPT9 to membranes is sufficient to recruit dynein and induce perinuclear membrane relocation. Biochemical assays using recombinant SEPT9 and native dynein-dynactin complexes revealed that SEPT9 interacts directly with both dynein and dynactin through its GTPase domain and N-terminal extension, respectively. Strikingly, SEPT9 preferentially associates with the adaptor-binding region of the dynein intermediate chain (DIC) in its GDP-bound state. Importantly, we found that under acute oxidative stress induced by sodium arsenite, SEPT9 localization to lysosomes is enhanced and SEPT9 is required for robust perinuclear lysosome clustering under stress. A SEPT9 GTPase-dead mutant, which is predicted to lock SEPT9 in its GTP-bound state, has significantly reduced dynein binding and fails to rescue perinuclear lysosome repositioning under oxidative stress. Collectively, our findings suggest that SEPT9 functions as a GDP-activated dynein adaptor/scaffold for the assembly of dynein-dynactin complexes on lysosomal membranes providing an alternative mechanism of retrograde lysosome transport at steady state and during cellular adaptation to stress.

Towards a quantitative understanding of intracellular dynamics during phagocytosis in vivo
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Dynamic cells need to adapt rapidly to environmental changes and coordinate different cellular activities. Microglia, the macrophages of the brain, are an excellent example of this. They shape neuronal connectivity by patrolling the central nervous system to identify, remove, and digest dying neurons and synapses. How microglia regulate these different activities is currently unclear. To address this, we have generated real-time reporters to visualize vesicular and cytoskeletal dynamics in the transparent zebrafish brain. By combining these reporters with cutting-edge light-sheet microscopy for in vivo brain imaging at single-cell resolution, we have uncovered key mechanisms underlying microglial behaviour and functionality. For example, we found that microglial migration and engulfment depend on efficient vesicular trafficking and phagosomal maturation in these cells. In addition, we discovered that microglia engulf dead neurons sequentially, despite being surrounded by several dead cells. In this context, the application of light-mediated chemical perturbations allowed us to uncover an essential role for microtubule dynamics in microglial decision-making and polarization towards these dying neurons. A better understanding these dynamic processes might shine a light on how microglia respond to changes in their environment, such as during neurodegeneration and disease.

Mitochondria and Calcium

Hypoxia-Induced Cardiomyocyte Mitochondrial Fission and Permeability Transition are Inhibited by Prostaglandin-Driven Bnip3 Phosphorylation
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Hypoxia-induced production of Bcl-2-like 19kDa-interacting protein 3 (Bnip3) plays a prominent role in cardiomyocyte necrotic cell death, disrupting subcellular calcium homeostasis and initiating mitochondrial permeability transition (MPT). Emerging evidence suggests both a cardioprotective role for protein kinase A (PKA) through stimulatory prostaglandin (PG) E1 signalling during prolonged periods of hypoxia, and a cytoprotective role for Bnip3 phosphorylation, indicating that post-translational modifications of Bnip3 may be a point of convergence for these two protective pathways. Using a combination of rodent hypoxia models, human iPSC-derived cardiomyocytes, rat primary neonatal cardiomyocytes (PVNCs), H9c2 cells, and genetically engineered mouse embryonic fibroblasts (MEFs) we tested if the PGE1 analogue misoprostol is cardioprotective by altering the phosphorylation status of Bnip3, inhibiting its ability to induce cardiomyocyte mitochondrial dysfunction and cell death. Here we report that acute hypoxia exposure significantly increases Bnip3 expression, mitochondrial-fragmentation, -ROS, -calcium accumulation and -permeability transition, while reducing mitochondrial membrane potential, all of which were restored to control levels with the addition of misoprostol, despite elevated Bnip3 protein expression. Through both gain- and loss-of-function genetic studies we further show that the misoprostol-induced protection directly affects Bnip3, preventing mitochondrial perturbations. We demonstrate that this is a result of PG EP4 receptor signalling, PKA activation, and direct Bnip3 phosphorylation at threonine-181. Furthermore, when this PKA phosphorylation site within Bnip3 is neutralized, the protective misoprostol effect is absent. We also provide evidence that Bnip3 phosphorylation enhances its interaction with 14-3-3β, which traffics Bnip3 away from the ER, thereby preventing aberrant ER calcium release and MPT. In vivo studies further demonstrate that misoprostol treatment increases Bnip3 phosphorylation at threonine-181 in the mouse heart, preventing hypoxia-induced reductions in cardiac ejection fraction and fractional shortening, which was also observed in Bnip3 knockout mice. Taken together, our results demonstrate a foundational role for Bnip3 phosphorylation in the molecular regulation of cardiomyocyte metabolic dysfunction and identifies a pharmacological mechanism, through PKA, to prevent hypoxia-induced cardiac injury.

P60

Mitochondria-lysosome contacts regulate mitochondrial Ca²⁺dynamics via lysosomal TRPML1

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Mitochondria and lysosomes are critical for cellular homeostasis, and dysfunction of both organelles has been implicated in numerous diseases. Recently, interorganelle contacts between mitochondria and lysosomes were identified and found to regulate mitochondrial dynamics. However, whether mitochondria-lysosome contacts serve additional functions by facilitating the direct transfer of metabolites or ions between the two organelles has not been elucidated. Here, using high spatial and temporal resolution live-cell microscopy, we identified a role for mitochondria-lysosome contacts in regulating mitochondrial calcium dynamics through the lysosomal calcium efflux channel, transient receptor potential mucolipin 1 (TRPML1). Lysosomal calcium release by TRPML1 promotes calcium transfer to mitochondria, which was mediated by tethering of mitochondria-lysosome contact sites. Moreover, mitochondrial calcium uptake at mitochondria-lysosome contact sites was modulated by the
outer and inner mitochondrial membrane channels, voltage-dependent anion channel 1 and the mitochondrial calcium uniporter, respectively. Since loss of TRPML1 function results in the lysosomal storage disorder mucolipidosis type IV (MLIV), we examined MLIV patient fibroblasts and found both altered mitochondria-lysosome contact dynamics and defective contact-dependent mitochondrial calcium uptake. Thus, our work highlights mitochondria-lysosome contacts as key contributors to interorganelle calcium dynamics and their potential role in the pathophysiology of disorders characterized by dysfunctional mitochondria or lysosomes.

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**Investigating the role of ER-mitochondria junction on store-operated Ca\(^{2+}\) entry**

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Store-operated Ca\(^{2+}\) entry (SOCE) is the major Ca\(^{2+}\) influx in not-excitable cells, which is activated when the Ca\(^{2+}\) level inside ER is low. Numerous studies revealed that mitochondria are also regulator of SOCE. However, the exact mechanism is still unclear. The percentage of mitochondria that contact with ER is around 5-20. This ER-mitochondria connection and the ability of ER to spatially and temporally coordinate with plasma membrane may allow mitochondria to distribute to the ER/PM contact region. We therefore hypothesize that mitochondria may regulate SOCE by their contacts with ER. We established real-time Ca\(^{2+}\) monitoring platforms, which can simultaneously demonstrate local Ca\(^{2+}\) dynamics in cytosol, mitochondria and/or endoplasmic reticulum (ER), by stably expressed genetic-encoded Ca\(^{2+}\) indicators (GECIs) in HEK293T and live cancer cell line. To elucidate the role of ER-mitochondria junctions (EMJs) in whole cell Ca\(^{2+}\) homeostasis, we broke EMJs by knocking down the tethering proteins, mitofusin2 (MFN2) and PDZD8 in our platform. EMJ breakdown not only disrupted the Ca\(^{2+}\) flows from ER to mitochondria, but also suppressed the Ca\(^{2+}\)-release activated Ca\(^{2+}\) entry (CRAC). Double knockdown of the tethering protein MFN2 or PDZD8 and SOCE components STIM1 and/or ORAI1 eliminated the effects of MFN2 or PDZD8 knockdown. Using pharmacological tools to slightly reduce ER Ca\(^{2+}\) storage didn’t affect SOCE activity. Furthermore, the expression level of ORAI1 and STIM1 didn’t significantly change in MFN2- or PDZD8- knockdown cells. To further explore the mechanisms of how EMJ may regulate SOCE, we used FRB-FKBP system to tether different organelles or proteins, such as ER, mitochondria, plasma membrane (PM) and ORAI1 protein. We noticed that tethering mitochondria to ER or PM upregulated SOCE in both MFN2 and PDZD8 knockdown cells. We also examined whether mitochondria distribute to ER/PM junctions using a membrane-attach-peripheral ER (MAPPER) marker and mitochondrial mCherry. Indeed, induce ER Ca\(^{2+}\) leakage to activate SOCE render mitochondria to translocate to ER/PM junctions. Our findings indicated that mitochondria may contribute to STIM1/ORAI1-mediated SOCE via EMJ. We are now investigating the molecular mechanisms of the above phenomena, which will revolutionize our understanding of mitochondrial Ca\(^{2+}\)-SOCE regulation and identify candidate proteins in the process.

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**The EF hand-like motif of Num1 facilitates the hierarchical integration of mitochondrial and nuclear positioning pathways**

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Organelle inheritance is highly regulated to ensure all cells receive a full complement of organelles during cell division. In *Saccharomyces cerevisiae*, the plasma membrane protein Num1 is required for the positioning and inheritance of both the nucleus and mitochondria. More specifically, Num1 tethers mitochondria to the cell cortex to allow for proper distribution and retention of mitochondria throughout the cell cycle. Num1 also anchors dynein at these cortical sites, which facilitates dynein-mediated spindle positioning during nuclear inheritance. We have shown that these two functions of Num1 are coupled, such that mitochondria drive the assembly of the cortical clusters that anchor dynein. To understand the contribution of mitochondria to cortical dynein anchoring, we developed a synthetic clustering system to form clusters of Num1 independent of mitochondria that can still tether mitochondria. Using this system we have found that dynein preferentially interacts with mitochondria-associated synthetic clusters. To further dissect this mitochondrial-dependent dynein anchoring, we took a structure-function approach and systematically truncated Num1 in our synthetic clustering system. We found that the EF hand-like motif (EFLM) of Num1 is important for the mitochondrial-dependent association of dynein with Num1. When the EFLM is deleted from Num1 in our synthetic clustering system, we find a dramatic increase in non-mitochondrial associated cortical dynein. We also observe similar results when we mutate residues in the EFLM of Num1 that are predicted to bind small molecules, such as Ca2+. These data suggest that the EFLM promotes an arrangement of Num1 that favors dynein anchoring in the presence of mitochondria and, consequently ensures dynein-mediated nuclear inheritance occurs after mitochondrial inheritance. Furthermore, when the EFLM is deleted in an otherwise wild type background, these cells no longer exhibit defects in dynein-mediated spindle positioning when mitochondrial inheritance is inhibited. These data suggest that the EFLM is required for the coupling of mitochondrial-dependent dynein anchoring and dynein-mediated spindle positioning. We hypothesize that this integration of mitochondrial and nuclear positioning pathways provides a spatiotemporal mechanism to ensure mitochondria are inherited prior to the nucleus and the subsequent completion of the cell cycle.

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The role of IGFBP-3 on mucosal epithelial cell mitochondrial homeostasis in hyperosmolar stress

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Purpose: Hyperosmolar stress is known to cause significant damage to mucosal epithelial cells in many disease states through an increase in reactive oxygen species, mitochondrial damage, and cell death. Prior work in our laboratory suggests that the insulin like growth factor binding protein 3 (IGFBP-3) is involved in the regulation of mitochondrial homeostasis in mucosal epithelia. The purpose of this study is to determine the role of IGFBP-3 in mucosal epithelial cells exposed to hyperosmolar stress. Methods: Telomerized human corneal epithelial (hTCEpi) cells and human bronchial epithelial cells (HBECs) were cultured in serum-free keratinocyte basal media (KBM) at 330 mOsm (control) or supplemented with NaCl to 450 mOsm (hyperosmolar). Cells were then incubated in the presence and absence of recombinant human (rh)IGFBP-3 for 2, 6 or 24 hours. Mitochondrial fractionation, western blotting, transmission electron microscopy, immunofluorescence and live cell fluorescent imaging were used to determine the expression of mitochondrial and mitophagy proteins and assess mitochondrial morphology. A Seahorse Metabolic Flux Analyzer was used to measure metabolic activity. Results: For
both cell types, 24 hours of hyperosmolar culture decreased intracellular and extracellular levels of IGFBP-3. In hTCEpi cells, there was a decrease in respiration and glycolysis that was associated with a shift towards a respiratory phenotype at 24 hours and an increase in mitochondrial calcium. As early as 2 hours, there was a loss in mitochondrial membrane polarization and an increase in mitophagic flux. After 6 hours of hyperosmolar culture, there was increased autophagy, an increase in mtDNA, and cell cycle arrest at G2/M. Co-treatment with rhIGFBP-3 blocked autophagy and the increase in mtDNA. For both cell types, at 24 hours, hyperosmolar culture showed ballooned mitochondrial with missing cristae. Co-treatment of cells with rhIGFBP-3 blocked the hyperosmolar-induced damage and instead demonstrated enlarged mitochondria with intact cristae. Conclusions: These data support a novel role for IGFBP-3 in mitochondrial homeostasis during hyperosmolar stress. Additional studies are needed to define the exact mechanism(s) by which IGFBP-3 regulates mitochondrial homeostasis in mucosal epithelia during stress.

New Techniques in Light and Electron Microscopy

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Ribosome-Associated Vesicles: a dynamic sub-compartment of the endoplasmic reticulum in secretory cells

The endoplasmic reticulum (ER) is a highly dynamic network of membranes. Here, we combine live-cell microscopy with in situ cryo-electron tomography (cryo-ET) and correlative light and electron microscopy (cryo-CLEM) to directly visualize ER dynamics in several secretory cell types including pancreatic beta cells and neurons under near-native conditions. Using these imaging approaches, we identify a novel, mobile form of ER, Ribosome-Associated Vesicles (RAVs), found primarily in the cell periphery, that is conserved across different cell types and species. We show that RAVs exist as distinct, highly dynamic structures separate from the intact ER reticular architecture that interact with mitochondria via direct intermembrane contacts. These findings describe a new ER sub-compartment within cells.

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Deep learning enables label-free detection of cell division in phase contrast microscopic time-lapses
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Label-free detection of cell division from two-dimensional phase contrast microscopy would allow for fine-grained monitoring of cell growth dynamics in many biological fields. Current cell cycle identification relies on fluorescent labels such as Fluorescent Ubiquitination-based Cell Cycle Indicator (FUCCI). While used in countless biological discoveries, there is mounting evidence that fluorescent sensors can alter biological responses stressing the need for label-free approaches. Although there are sophisticated label-free imaging technologies, such as quantitative phase contrast microscopy, simple bright-field and phase contrast imaging remains the most widespread technologies due to their availability and ease-of-use. Thanks to great advances in deep learning (DL)-based image processing in recent years, label-free cell segmentation and cell migration tracking are now feasible even in low-contrast phase contrast microscopy images. Typical DL-algorithms are however very data-hungry, meaning that large quantities of annotated data are required to retrain such system for new use-cases or cell types. We present a new data-efficient algorithm to detect cell division in phase contrast microscopy time-lapses combining existing methods for label-free cell segmentation with temporal cycle consistency learning, a type of DL-method for video analysis. Using as little as 70 hand-picked video-examples of single cells dividing, our algorithm learns to detect cell division with more than 90 % accuracy without any explicit annotation of when division occurs in the videos. Thanks to its data-efficiency and hands-off learning, our algorithm can be easily customized for new cell types and treatments. We demonstrate its performance in high-throughput live cell imaging and validate our results by comparing to fluorescent FUCCI labels on AU565-cells treated with drugs impacting rate and synchronization of cell division. Our algorithm provides a reliable and easily adaptable method to detect, characterize and quantify cell division in fine-grained studies of cell growth dynamics.

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**Light microscopy of proteins in their ultrastructural context**

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Fluorescence microscopy has revolutionized the field of cell biology through its exceptional contrast and high specificity of labeling. With the advent of super-resolution microscopy, the three-dimensional (3D) distribution of proteins of interest can be imaged at spatial resolutions down to ~10 nm, revealing astounding sub-cellular organization at the nanoscale. Showing these proteins in the ultrastructural context of the cell, however, has so far relied on slow and highly specialized correlative microscopy techniques that combine the resolving power and global contrast of electron microscopy (EM) with the information provided by fluorescence microscopy. In this work, we report the discovery of a new principle for an optical contrast equivalent to electron microscopy (EM) which reveals the ultrastructural context of a cell with a conventional confocal microscope. By decrowding the intracellular space through 13 to 21-fold physical expansion while simultaneously retaining the proteins, bulk (pan) labeling of the proteome resolves local protein densities and reveals the cellular nanoarchitecture by standard light microscopy [1]. We resolve for the first time with a light microscope (1) two sides of a single mitochondrion cristae, (2) individual Golgi cisterna, and (3) individual synaptic vesicles in cultured neurons. Our method, which we named pan-ExM, has the potential to expand and democratize ultrastructure imaging and thus transform the field of cell biology. [1] M'Saad, O. & Bewersdorf, J., *Nature Communications*, in press (2020)
Lung organoids as an assay model for in vitro assessment of toxicity effects to lungs using high content imaging and 3D image analysis.

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Organoid models gain increasing popularity in biologic research and disease modelling to re-capitulate complexity of real tissues. To model the in vivo human lungs, we have cultured primary human airway epithelial cells under conditions that promote the formation of 3D structures recapitulating the morphological and functional characteristics of the airway. In lung organoid cultures, epithelial cells were cultured in ECM supplemented with a mixture of growth factors. The organoids then self-organize into complex structures retaining clusters of multi-lineage epithelial cells. These special characteristics made organoid culture a promising system for a wide range of applications in both basic and translational approaches such as drug screening, and disease modeling. High content imaging allows monitoring and quantitation of growth and differentiation of lung organoids, 3D reconstitution of the structures, and complex analysis of organoids structure, cell morphology and viability, as well as expression of different cell markers. We describe the methods for confocal imaging and 3D analysis for lung organoids that allows visualization and quantitation of the number, size distribution, cell number, cell content, cell viability, volume, as well as quantitation of cell proliferation and expression of specific markers. We characterized multiple quantitative descriptors of organoids that could be used for studying disease phenotypes and compound effects. We demonstrate concentration-dependent effects of several drugs that been known to cause lung toxicity (ibrutinib, imatinib, doxorubicin) on spheroids morphology, growth, and cell viability. We demonstrated that concentration responses to cytotoxic compounds were significantly different between 3D and 2D cultures, which highlights the importance of using more complex models for evaluation of compound effects. In addition, we evaluated cell responses to stimulation with inflammatory cytokines. The assay can be used for toxicity evaluation of chemicals, drug screening, or disease modelling.

A correlative workflow for electron microscopy and atomic force microscopy provides a detailed analysis of the functional association between composition and sample properties for biomaterials.

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Atomic force microscopy (AFM) has a range of different imaging modes that provide information on the physical properties of biological specimens (1), operating in both dry and “wet” liquid environments to study dynamic processes. Electron microscopy (EM) provides ultrastructural information at high resolutions and can be combined with energy dispersive x-ray spectrometry (EDS) for compositional information (2) but can only examine samples that have undergone some level of sample fixation and stabilisation for the EM environment. While both microscopy techniques offer valuable information on biological samples, correlative approaches pose new challenges in the acquisition and analysis of data, particularly where there is a need for information on the same specimens and regions of interest (ROI). A workflow was produced to incorporate sample preparation, moving the sample between microscopes, collecting data on the same ROI accounting for significantly different scales of magnification and fields of view, and correlating very different sets of data following acquisition. This was conducted on tissue
samples to investigate the integration and degradation of hydroxyapatite bone implants in a minipig model. Tissue samples were fixed, embedded in resin, and ground to a thickness of approximately 1mm before being mounted onto an aluminium SEM stub and stained using uranyl acetate and lead citrate. The samples were coated in approximately 10nm of carbon. The sample was imaged using SEM first and a large area map was captured, composed of a montage of 240 images containing EM and EDS information. The sample was then transferred to an AFM for measuring materials properties of the sample with the large area map data used as a guide to the identification of ROIs. Data was correlated using Relate software, revealing areas where the composition of the implant and the measured materials properties showed correspondence. As correlative microscopy approaches become standard for biological research, it is important that workflows incorporating different types of imaging technology area established for a range of specimen types so that researchers can make informed decisions about sample preparation and imaging hierarchy. (1) Dufrêne, Yves F., et al. "Imaging modes of atomic force microscopy for application in molecular and cell biology." Nature nanotechnology 12.4 (2017): 295-307. (2) Scotuzzi, Marijke, et al. "Multi-color electron microscopy by element-guided identification of cells, organelles and molecules." Scientific reports 7.1 (2017): 1-8.

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Scalable ultrastructural mapping and characterisation of liver organoids using high resolution field emission scanning electron microscopy

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Background and Aim: Organoid biology is a rapidly growing field that has emerged as a product of our understanding of stem cell fate initiation and cellular differentiation. Organoids are three-dimensional (3D) in vitro cell cultures containing several cell types that develop from stem cells or organ progenitors and self-organise through cell sorting and spatially restricted lineage commitment, similar to organogenesis in vivo. These cell cultures can be used to model organogenesis, tissue homeostasis and disease response in vitro. The past decade has seen the emergence of a new era for volume imaging with novel microscopic approaches that can cross scales from cell to tissue, which has been essential to probe the complexity of organoid biology. The objective of the present study was to achieve wide-view liver organoid ultrastructure imaging using innovative scanning electron microscopy applications. This would allow us to examine the key features of cellular morphology within the context of the entire tissue culture. Methods: We applied High Resolution Field Emission Scanning Electron Microscopy (HRSEM) of ultrathin epoxy sections and low kV scanning transmission electron microscopy (STEM) detectors to generate transmission electron microscopy-like (TEM-like) images. This was to examine the cellular ultrastructure and morphology and assess the similarity of liver organoids to intact liver tissue. This novel approach was combined with automated scan generation and image tiling to generate mesoscale ultrastructural maps covering the entire organoid area. Results: By applying HRSEM/STEM we were able to image entire liver organoid over 100 μm in diameter with clearly definable cell ultrastructure at ~5 nm resolution. Key features of cellular morphology we observed were; the nucleus, heterochromatin, microvilli, mitochondria, lysosomes, exosomes, tight-junctons, the bile canaliculus and golgi complex. We also observed cell autophagy and apoptosis in the context of larger tissue structures. This was used to observe the liver parenchyma cell ultrastructure in our models of liver injury. Conclusion: We conclude that entire organoid ultrastructure imaging can be achieved using
HRSEM/STEM. We propose that the generation of ultrastructure imaging of entire liver organoid at <10 nm per pixel resolution is key to characterize cell structure and organelles with the prospects of assessing drugs that target various pathologies including liver fibrosis.

Non-Muscle Myosin

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Non-muscle myosin-2 contractility-dependent actin turnover limits the length of epithelial microvilli
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The apex of transporting epithelial cells is covered with microvilli, which form a surface of interaction with the luminal contents. In the context of the intestinal tract, brush border microvilli drive functions that are critical for physiological homeostasis, including nutrient uptake and host defense. However, cytoskeletal mechanisms that regulate the assembly and morphology of these protrusions are poorly understood. The parallel actin bundles that support microvilli have their pointed-end rootlets anchored in a highly crosslinked filamentous meshwork referred to as the “terminal web”. Classic electron micrograph studies hinted at the presence of a non-muscle myosin-2 (NM2) in the terminal web and postulated a role for this motor as a crosslinker of core actin bundles. We followed up on these classic studies by investigating if brush border morphology is impacted by compounds that inhibit or activate NM2. Strikingly, activation of NM2 with Calyculin-A resulted in microvillar shortening, whereas inhibition with Blebbistatin led to elongation; NM2 inhibition also dramatically reduced actin turnover in microvilli. To further understand which NM2 isoform mediates these effects, we turned to scRNAseq analysis of intestinal epithelial cells. This analysis revealed non-muscle myosin 2C (NM2C) is the most abundant isoform in villus enterocytes. Using super-resolution microscopy, we found that NM2C localizes in striking layer of uniformly spaced puncta at the level of the terminal web. Finally, overexpression of WT NM2C shortened microvilli, and expression of NM2C variants with activity blocking mutations attenuated this effect. Thus, NM2C uses its motor activity to control the dimensions of the actin bundles that support epithelial microvilli. These findings illuminate on the role of terminal web myosin and demonstrate a potential role for contractility-based actin turnover in apical morphogenesis.

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Permanent trapping of non-muscle myosin II in Drosophila neural stem cells
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Asymmetric cell division (ACD) is an important developmental process which is used to create cellular diversity and is conserved in bacteria, insects, mammals and plants. Defects in ACD may result in developmental defects, disease or cancer. ACD can take many forms to give rise to two daughter cells with different cell fates. One form is recognized as the difference in daughter cell size also known as sibling cell size asymmetry. Currently, the physiological role of sibling cell size asymmetry remains elusive. In Drosophila neural stem cells, sibling cell size asymmetry is influenced by changes in the localization of active non-muscle myosin II. Our objective was to begin examining how permanent changes to physical asymmetry influence cell fate. To manipulate sibling cell size asymmetry in
developing *Drosophila* brains, we permanently trapped active non-muscle myosin II, via myosin regulatory light chain, to the apical cortex using an anti-GFP nanobody (vhhGFP). Consistent with previous results, we found that apical myosin trapping can induce symmetric as well as inverted asymmetric neural stem cell divisions. Our initial analysis further suggest that altered sibling cell size does not correlate with cell cycle timing. Overall, these results begin to highlight the urgency in examining other factors which may be required for proper physical ACD in *Drosophila* neural stem cells. These attributes could conceivably be conserved with various organisms which undergo physical ACD. Future work will determine if changes to physical ACD can affect the fates of their progenitors which may result in abnormal neurogenesis.

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**Identifying molecular mechanisms of non-muscle myosin 2 filament amplification**

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How cells generate sufficient contractile forces to drive a diverse array of cellular processes is an outstanding question. Non-muscle myosin 2 is a motor protein that hydrolyzes ATP to contract actin filaments, generating the majority of contractile forces in non-muscle cells. Producing physiological levels of contraction requires many myosin 2 filaments. Recent work using high-resolution imaging demonstrated a model in which nascent myosin 2 filaments are amplified by an actin-dependent partitioning process - an alternative to the classic single filament assembly model. We believe this is a dominant myosin 2 assembly mechanism to rapidly drive physiological levels of contraction, yet we do not understand the molecular mechanism for this phenomenon. Two mechanisms have been proposed (1) Single-filament partitioning - A single myosin 2 filament (~30 monomers) is ripped in two as dynamic actin fibers separate. (2) Multi-filament partitioning - Two or more myosin 2 filaments (>>30 monomers) are separated from one another as dynamic actin fibers separate. To distinguish between these mechanisms, we are using complementary methods of molecular counting and correlative light and electron microscopy (CLEM). Our in vitro, fixed-cell, and live-cell imaging suggest that 1) in vitro single filaments are 30 monomers, 2) live-cell bipolar structures can be upward of a single filament, and 3) partitioning events appear to occur at or above that of a single filament. Recent imaging in the lab using an active-RhoA GTPase fluorescent biosensor revealed active RhoA localization following myosin cluster growth. This sequence of appearance - myosin cluster growth followed by RhoA accumulation - is unexpected as RhoA positively regulates myosin assembly. This suggests a possible feed-forward system where local increases in contractility from myosin clusters lead to RhoA activation, and thus further myosin assembly. Collectively, this work demonstrates that we can accurately quantify dynamic macromolecular complexes; using this technique we can better understand myosin 2 filament amplification and determine its dependence on a feed-forward RhoA activation pathway.

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**Trans-membrane protein traffics by loss of yeast type V myosin**

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Retrieval of cargo proteins from the endosome towards the trans-Golgi network (TGN) is a crucial intracellular process for cellular homeostasis. Its dysfunction is associated with pathogenesis of Alzheimer and Parkinson's diseases. Myosin family proteins are cellular motors walking along actin filaments by utilizing the chemical energy from ATP hydrolysis, known to involve in pleiotropic cellular trafficking pathways. However, the question of whether myosins play a role in the trafficking of Snc1 and Vps10 has not been addressed yet. The present study assesses the potential roles of all five yeast myosins in the recycling of two membrane cargo, Snc1 and Vps10. It appears that all myosins except Myo2 are not required for the Snc1 traffic, while it was found that Myo1 and 2 play important roles for Vps10 retrieval from the endosome and the vacuole. Multiple myo2 mutants harboring a point mutation in the actin binding or the cargo binding tail domain were characterized to demonstrate abnormal Vps10-GFP and GFP-Snc1 distribution phenotypes, suggesting severe defects in their sorting and trafficking at the endosome. Furthermore, Vps10-GFP patches in all tested myo2 mutants were found to be near stationary with quantitative live cell imaging. Finally, we found that actin cables in the myo2 mutant cells were considerably disrupted, which may aggravate the trafficking of Vps10 from the endosome. Together, our results provide novel insights into the function of Myo-family proteins in the recycling traffic of Vps10 and Snc1 destined for the TGN.

A Comparative Analysis of the Biochemical Properties of Mutant NMII-C1 (Myh14) Found in Human Oral Cancer Patients

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Non-muscle myosin II (NMII) proteins are cytoskeletal mechanoenzymes associated with cell morphology, migration and division. Genetic mutations in NMII paralogs (MYH9, 10 and 14) may compromise their Mg-ATPase activity, disrupt filament assembly or alter sub-cellular localization, and have been implicated in diseases as deafness, thrombocytopenia, autism and cancer. Samples collected from patients suffering from Gingivo-buccal Oral Squamous Cell Carcinoma (OSCC-GB), a subtype of HNSCC, showed six somatic mutations in MYH14 gene (Nat Commun 5, 5835 (2014)), whose implications in cancer progression are yet to be determined. We prepared the six mutants in NMII C1 (the major alternatively spliced isoform of NMII C found in tumour) background which are distributed across the head and tail domains of heavy chain of NMII C (encoded by MYH14 gene), and found that the NMII C1 mutants differentially affected the actin binding, and heteropolymer forming ability with NMII B. Tail domain mutations (Q890K, E1733Q and E1797D) showed increased binding with actin, and mutations in the actin-binding region (D597Y and S655R) of head domain showed increased in heterofilament formation with NMII B, as evidenced by pull-down assays. Triton X-100 soluble-insoluble assay showed that mutations in tail domain (Q890K, E1733Q and E1797D) and ATP-binding region of head domain (E291Q) disrupted actomyosin complex formation in an ATP-dependent and ATP-independent manner, respectively. FRAP analysis displayed significantly greater mobile fraction and faster fluorescence recovery rate with E291Q and E1797D compared to WT, in MDA-MB 231 cells. Thus, mutations in tail and ATP-binding head domain affect NMII C1 dynamics in the cells, which may have significant role in cancer progression.
Regulation of Nonmuscle Myosin II-C by miRNAs in Mouse 4T1 Cells

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Nonmuscle myosin II-C (NMII-C), one of the paralogs of NMII, plays important role in many cellular processes such as cytokinesis, adhesion, migration and mechano-sensitivity. Unlike NMII-A and II-B, NMII-C is relatively less abundant in majority of tissues and cell lines. Whereas expression of NMII-C is inducible in a variety of cell lines, the mechanism is yet to be explored. Here, we report a group of microRNAs (mmu-miR 200a*, mmu-miR 532-3p, mmu-miR 680 and mmu-miR 1901) that can regulate the expression of NMII-C both in silico and in vitro. All these microRNAs have canonical as well as non-canonical binding sites at 3'UTR and coding region of NMHCII-C mRNA, and each of them is able to downregulate NMHCII-C but at different degree as assessed by dual-luciferase assay and immunoblot analysis. Changing the NMII-C expression by any of these miRNAs in 4T1 cells shows an increase in cell migration, colony formation and tumor formation in allograft model. Altogether, this study demonstrates the probable mechanism of NMII-C regulation by endogenous miRNAs, and it also reveals a role of NMII-C in tumorigenicity.

Characterization of Nonmuscle Myosin 2 Functions in Mouse Cardiac Valve Development

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The structure composing the endocardial cushions develops into the cardiac septum and the cardiac valves during heart development. Nonmuscle myosin 2 (NM2) has important functions in cell migration and adhesion. Two paralogs of NM2 (NM2A and 2B) are expressed in the endocardial cushions. In order to study the role of NM2 in cardiac valve development, we ablated NM2 in endocardial cells, which contribute to the majority of the mesenchymal cells of the cardiac cushions during the endothelial-mesenchymal transition process. Mice ablated for either NM2A or NM2B alone from endocardial cells show no obvious defects in cardiac cushions. Mice ablated for both alleles of NM2B and one allele of NM2A demonstrate major defects in cardiac cushion remodeling in E14.5 embryos, resulting in congenital valvular defects. Mice ablated for both alleles of NM2A and one allele of NM2B show moderate defects in cardiac cushions. Upon further observation of the intact cardiac cushions following tissue fixation, clearing, and confocal microscopy, we found differences in the cell alignment between the wild-type and the ablated mice. While the wild-type mouse cushions develop into functional valves with clear cell alignment, the ablated cushions show chaotic cellular patterns. Consistent results were found in the mouse embryonic fibroblasts cultured in 3D collagen gels. The cells ablated for NM2B lose the directionality and alignment observed in the wild-type cells, implying that the lack of NM2B negatively affects cell migration and alignment, consequently leading to failed cushion remodeling. Our current findings demonstrate the important role of NM2 in cardiac valve development.
Deciphering the role of myosin contraction in mammalian cell migration velocity and energy output

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Cell migration in open spaces typically requires actin polymerization and myosin contraction. The myosin retracts the trailing edge of the cell and provides active contractile stress within the actomyosin network, which also prompts the depolymerization of the actin network. Experimentally it has been well established that inhibition of myosin leads to reduced cell velocity. Our previous work has suggested that modulating the active contractile stress alone does not affect cell migration. This imposes an interesting question as, given the multiple roles of myosin contraction, which exact mechanism contributes most to cell migration velocity, and how this factor influences the effective force generated by the cell. The goal of this study is to elucidate the detailed effects myosin contraction in cell migration velocity and the effective migratory energy output. With the current experimental techniques, it is still challenging to measure the stress within cells, the concentration of molecules, and the mechanical energy from live-cell experiments. We have thus developed a physiology-based mathematical model with four coupled species: G-actin, F-actin, inactivated myosin, and activated myosin. Each species has its own governing equation. In the model the actin polymerization happens at the leading edge and the depolymerization happens within the entire cell, as consistent with the current knowledge. The model predicts that the myosin contraction contributes to cell velocity via increasing the rate of actin depolymerization within the actin network, instead of through a direct change of the stress state inside the cell. The reduced actin network density due to excess depolymerization also reduces the adhesive force at the trailing edge of the cell. The combined effects of these two factors, as the model predicts, leads to a biphasic cell migration velocity as a function of the myosin contractility. The effective force generated by the cell can be represented by the power output of the cell. Interestingly, our model predicates that even if cells migrate at the same velocity (on the two sides of the biphasic velocity curve), the effective cell energy outputs are distinct: higher myosin contractility leading to higher migratory energy output at the same velocity. The model has suggested a mechanism that how myosin is involved in regulating cell velocity. The cell migratory power output is an indication that how much physical barrier a cell can overcome during migration. Our results indicate that cell velocity alone does not determine the effective power output of cell migration. The work has implications on how cells in vivo design the intracellular machinery to achieve desired migration outcome.

Blebbing and Lamellipodia Depend on Nonmuscle Myosin II Activity

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Cells like cancer cells can adopt mesenchymal and amoeboid mode of migration in the neighbouring environment through membrane protrusive activities, lamellipodia and blebbing, respectively. Membrane protrusive activity is dependent on the activity of small GTPases Rac and Rho, which control
actin polymerization, actomyosin contractility, and cell-matrix adhesion. It is unclear how myosin II activity is regulated in these membrane protrusive activities and their interconversion, which in turn can modulate the mode of migration. Here we show that addition of ROCK inhibitor, Y27632, induces transition from blebbing to lamellipodia whereas addition of lower dose of MLCK inhibitor, ML7 induces lamellipodia to blebbing conversion in MDA-MB-231 human breast cancer cells. Interestingly, addition of (-) blebbistatin at >IC50 value inhibits both membrane protrusive activities whereas at IC50 (2µM), transition occurs from blebbing to lamellipodia, suggesting the association of different degree of NM II activity in these processes. Similar observations were seen in other cell lines like MCF-7 and MCF10A, albeit with different doses of inhibitors. Altogether, this study reveals that the amount of myosin II activity, which is differentially activated by ROCK and MLCK, is a critical parameter for a cell to exhibit blebbing or lamellipodia.

Organelle and Metabolic Regulation of Aging

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Worms treated with an ethanolic extract from *Artemisia scoparia* are fatty but live long

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Worms treated with an ethanolic extract from *Artemisia scoparia* are fatty but live longLike other biological processes, aging is not random, but subject to molecular control. Several conserved longevity pathways, including the insulin/IGF-1/FOXO pathway, have been shown to regulate lifespan across animal species. In principle, natural products that act on these pathways may be potent candidates for promoting healthy aging and longevity. But, few natural products capable of extending animal lifespan by a significant percentage have been identified to date. Previously, a botanical extract from *Artemisia scoparia* (SCOPA) was shown to have metabolically - beneficial effects on insulin action in mice. Here, we tested if SCOPA would have pro-longevity effects, using the nematode *Caenorhabditis elegans* as a model organism. We have found that wild-type *C. elegans* treated with 500 µg/ml SCOPA live ~40% longer than control animals. This lifespan extension is accompanied by striking physiological changes. First, SCOPA-treated worms appear short and stocky throughout life. Although they move slower than controls in younger ages, they preserve a healthy appearance and movement capacity later into life. Additionally, SCOPA-treated worms show a darker coloration, which is maintained even in old age. This coloration appears to be linked to higher fat storage, consistent with reports that SCOPA inhibits lipolysis in mice and enhances lipid accumulation in adipocytes. Notably, we have found that SCOPA-mediated lifespan extension and fat accumulation require DAF-16, the *C. elegans* FOXO transcription factor. In SCOPA-fed animals, DAF-16 translocates from cytoplasm to nucleus, indicating that this botanical extract may bring about metabolically - advantageous effects by modifying DAF-16 target-gene expression. Indeed, we have evidence that the expression of some DAF-16 target genes involved in fatty-acid metabolism and biosynthesis is upregulated in SCOPA-treated animals, in line with previous reports that over-expression of "fat" genes can increase *C. elegans* lifespan. Together, our data indicate that SCOPA-treated worms are fatty but metabolically healthy, and that they may live long due to altered insulin signaling.
P80

**Novel bacterial diets as nutraceuticals to alter lifespan and healthspan trajectories in C. elegans**

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Diet is one of the more variable aspects in life due to the variety of options that organisms are exposed to in their natural habitats. In the laboratory, *C. elegans* are raised on bacterial monocultures, traditionally the *E.coli B* strain OP50, and spontaneously occurring microbial contaminants are removed to limit experimental variability because diet - including the presence of contaminants, can exert a potent influence over animal physiology. In order to diversify the menu available to culture *C. elegans* in the lab, we have isolated and cultured three such microbes: *Methylobacterium*, *Xanthomonas*, and *Sphingomonas*. The nutritional composition of these bacterial foods are unique, and when fed to *C. elegans*, can differentially alter multiple life history traits including development, reproduction, and metabolism. In light of the influence each food source has on specific physiological attributes, we comprehensively assessed the impact of these bacteria on animal health and devised a blueprint for utilizing different food combinations over the lifespan, in order to promote longevity. The expansion of the bacterial food options to use in the laboratory will provide a critical tool to better understand the complexities of bacterial diets and subsequent changes in physiology and gene expression.

P81

**Effect of oxidative stress on mitochondrial function in primary human trabecular meshwork cells**

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**Introduction:** Pseudoexfoliative glaucoma (PXFG) is form of open angle glaucoma (OAG) secondary to the accumulation of extracellular exfoliation material in the human trabecular meshwork (HTM). Oxidative stress, intracellular reactive oxygen species (ROS), and mitochondrial damage have been implicated in PXFG. The objective of this study was to determine the mitochondrial membrane potential ($\Delta \Psi_m$) in HTM cells after ROS exposure, as a measure of mitochondrial function. **Methods:** Primary (isolated from 2 donors) HTM cells were routinely cultured in DMEM/F12 supplemented with 10% fetal bovine serum and antibiotics. Cells were plated at 25,000 cells/cm², allowed to attach overnight. Cells were treated with 500 μM H₂O₂ in serum free media for ~4 hours, while control cells were treated with serum free media. After treatment, they were stained with JC1 and imaged. JC1 fluorescence shifts from an emission peak of ~530 nm (green) to ~590 nm (red) as an indicator of $\Delta \Psi_m$. We quantified $\Delta \Psi_m$ as the ratio of red/green emission. **Results:** Analysis of the imaged cells reveals that H₂O₂ treatment resulted in a decrease of the $\Delta \Psi_m$ in both cells from both donors. Red/green ratio of treated cells was 25.1% and 84.5% of control values for the two donors. These results indicate H₂O₂ acutely decrease mitochondrial function. Future work will include additional measures of mitochondrial function and damage, as well as consider chronic ROS exposure. **Acknowledgements:** This work was supported by a Grant-In-Aid from The Glaucoma Foundation.
An analysis of ceramide and lipid metabolism in aging C. elegans
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The increase in our aging population in combination with the growing incidence of late-life conditions including dementia, cancer, and diabetes, continues to burden our healthcare system and presents a need for research on health aging. Here, we present work examining the roles of sphingolipid metabolism in aging. Sphingolipids play an important role in stress response, cell survival, cell signaling, and aging. Specifically, the sphingolipid, ceramide, responds to oxidative stress and recruits apoptotic proteins to the cell membrane thereby causing cell cycle arrest. Furthermore, ceramide levels increase with age, making it an important lipid mediator of aging. The objective of this study is to examine the physiological role of ceramide aging and lipid metabolism pathways in the roundworm, Caenorhabditis elegans. To assess these mechanisms, we utilized C. elegans’ mutants lacking acid sphingomyelinase (asm-3) and ceramide synthase (hyl-2) which we previously found to be long- and short-lived respectively. We wanted to explore longitudinal changes in lipid concentrations in aged asm-3, hyl-2, and wildtype (N2) worms. To do this, we collected approximately 600 animals at 1, 5, and 10 days old and analyzed them using mass spectroscopy. Among our samples, approximately 700 different lipids were abundant enough in concentration for analysis. Interestingly, at 10 days old, hyl-2 mutants, which have a reduced life-span showed an increased concentration of eicosapentaenoic acid (EPA), and asm-3 mutants, which are long-lived animals, have reduced levels of EPA. To expand upon our lipidomic data with enzymatic findings, we are utilizing RT-qPCR analysis to longitudinally analyze stress response genes including superoxide dismutase (sod-3), fatty acid desaturases (fat-4 and fat-1) and fatty acid tail elongases (elo-5 and elo-6) in 1, 5, and 10 day old hyl-2 and asm-3 mutants. Furthermore, to expand upon the current knowledge of ceramides’ role in lifespan, we present data on the suppression of hyl-2 by RNAi in long-lived mutants including coenzyme Q7 (clk-1), insulin like signaling (daf-2), and dietary starvation (eat-2). With these assays, we hope to better understand the intrinsic biochemical lipid processes associated with ceramide metabolism in long-lived animals.

The mitochondrial-derived peptide MOTS-c restores homeostasis in aged human placenta-derived mesenchymal stem cells in vitro
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The mitochondrial-derived peptide MOTS-c restores homeostasis in aged human placenta-derived mesenchymal stem cells in vitro
Mesenchymal stem cells (MSCs) are multipotent cells with critical roles in homeostasis and regeneration. MSCs undergo aging in response to various stresses, and this causes many diseases including regenerative disorders. Thus, regulation of aging factors is critical for healthy aging. Mitochondrial open reading frame of the 12S rRNA-c (MOTS-c) was recently reported to regulate metabolic homeostasis. Here, we investigated the restorative effects of MOTS-c on aged human placenta-derived MSCs (hPD-MSCs). MOTS-c restored the morphology of old hPD-MSCs. MOTS-c significantly activated AMP-activated protein kinase, which is the main target pathway of MOTS-c, and inhibited its antagonistic effector mTORC1. MOTS-c considerably restored mitochondrial homeostasis by
decreasing oxygen consumption and reactive oxygen species production. The mitochondrial state of MOTS-c-treated old hPD-MSCs was more similar to that of young hPD-MSCs than the mitochondrial state of non-treated old hPD-MSCs. MOTS-c decreased lipid synthesis. In conclusion, we demonstrated that MOTS-c restores homeostasis in aged hPD-MSCs.

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**A novel aging determinant regulates the actin cytoskeleton, nutrient sensing, and lifespan in yeast.**

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In yeast, actin cables are bundles of actin filaments that serve as tracks for polarized cargo movement from mother cells to buds and are essential for cell division. Beyond its roles in cargo transport and cell division, our previous studies revealed that the actin cytoskeleton can directly impact lifespan. Actin cables undergo retrograde flow and treadmilling from buds towards mother cells, and in doing so promote the fitness and lifespan of daughter cells by acting as filters to prevent movement of low functioning mitochondria from mother cells to developing daughter cells. Similar to other organelles, the actin cytoskeleton exhibit declines in aging in many eukaryotes, and we have observed similar age-associated declines in actin cable stability and function in yeast. However, the mechanism underlying these declines remains largely unknown. Therefore, we performed a genome-wide screen to identify novel genes required for actin function when exposed to the actin destabilizing agent, Latrunculin-A. We identified a previously uncharacterized open reading frame (ORF), which when deleted, increases actin cable stability and abundance, promotes mitochondrial fitness, and extends replicative lifespan. Our transcriptome analysis revealed an unexpected role for the ORF in regulating branched-chain amino acid (BCAA) metabolism and abundance. Moreover, we found that mutations that alter BCAA metabolism or depletion of specific BCAAs produce effects on actin cables that are similar to those observed upon deletion of the ORF. Although the TORC1 pathway has established functions in sensing amino acids and regulating lifespan, the observed effects of the ORF on BCAAs are independent of TORC1. Overall, we discovered 1) a novel regulator of BCAA metabolism and the actin cytoskeleton, 2) a novel role for BCAA in control of the actin cytoskeleton, and 3) a TORC1-independent mechanism for BCAA control of mitochondrial and cellular fitness and of lifespan control. Our studies highlight the connection between nutrients, the actin cytoskeleton, and longevity and provide a foundation for understanding how aging targets the actin cytoskeleton and conversely, how the actin cytoskeleton impacts lifespan control.

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**Metabolic Regulation of the Mitochondrial-Derived Compartment Pathway**

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Amino acids are an integral part of our nutrition and are necessary for cell proliferation, regulation of gene expression and protein synthesis. However, recent studies have linked elevated levels of amino
acids, particularly branched-chain amino acids (BCAAs) to mitochondrial dysfunction associated with a host of metabolic and age related disorders. How amino acids impair mitochondrial function and the mechanisms cells utilise to detoxify amino acids remains largely unknown. We recently discovered a new cellular structure conserved from yeast to mammals that is activated by elevated levels of amino acids, called the Mitochondrial-Derived Compartment (MDC). MDCs are large, micron-sized membrane-bound structures that are generated from mitochondria at sites of ER-mitochondrial contact. Upon formation, MDCs selectively remodel the mitochondria tubule, sequestering outer mitochondrial membrane proteins as well as inner membrane metabolite carriers from the organelle while leaving the rest of the mitochondrion intact. MDCs form in response to high levels of amino acids within the cells, with branched chain amino acids, particularly leucine, being the most potent inducer. Our working model is that the MDC pathway functions as a mechanism to control levels of mitochondrial nutrient transporters in response to changes in cellular nutrient status. Currently, a major unanswered question surrounding the MDC pathway is how amino acid cues are relayed to activate the pathway. To address this, we analyzed the role of common nutrient sensing pathways in MDC formation. We found that canonical amino acid sensing pathways were not required for MDC formation. However, we did identify a requirement for Cdc60 (leuRS), a leucyl tRNA synthetase and known leucine sensor for the mTOR pathway, in MDC formation. We are currently exploring the role Cdc60 plays in the MDC pathway and testing whether it serves as a key sensor for relaying leucine status to the MDC pathway.

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The effects of aging & hyperglycemia on Ca metabolism in zebrafish scales
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Bone metabolism is maintained by remodeling the cycle of formation by osteoblast and resorption by osteoclast. Imbalance of these processes causes osteoporosis. There are many patients around the world, and effective therapeutics are required. This study aims to establish new in vivo models that promote the understanding of osteoporosis and are useful for drug screening. Osteoporosis is related to some factors such as aging and diabetes. Aging causes changes in hormone balance and reduction of Ca intake, which make the cycle out of balance in human. Diabetes is one of the causes of osteoporosis as well. Low insulin secretion or sensitivity causes not only hyperglycemia but also low osteoblast activity and low bone formation. In this research, we used zebrafish scales as a model to study Ca metabolism. They have osteoblast and osteoclast cells like human bones and are maintained by remodeling. Moreover, because scales exist outside of the body, neither high skills nor killing animals is required. In this research, we conducted two experiments. In the first experiment, we divided zebrafish into three groups by their age. In the second experiment, we induced diabetes by using alloxan which inhibits insulin release. We measured the area of scales, osteoblast and osteoclast activities, Ca to P molecular ratio (Ca/P) which shows the mineralization level whose decrease means that the scales are fragile, and also blood glucose levels in the second experiment. In the first experiment, we found that in 19-31-month-old zebrafish which are considered as aged, although their osteoclast activity was higher than in 4-7-month-old zebrafish, the scale area was larger than that of 4-7-month-old zebrafish, and Ca/P was higher as compared to those of 4-7 and 9-15-month-old zebrafish. These results indicate that the scales of 19-31-month-old zebrafish had more mechanical strength than the other two groups. In the second experiment, blood glucose levels of the alloxan group was higher than those of the control group as
expected, and the area, osteoblast activity, and Ca/P decreased in the alloxan-treated group. These results indicate that the scales of alloxan-induced zebrafish had less mechanical strength. Taken together, our data suggested that in the zebrafish scale assay, aging did not cause osteoporosis at least until 31 months old, but diabetes caused osteoporosis and can be used as in vivo model.

Organelle Dynamics in Neurons

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Mitochondrial Maintenance in Neurons
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Neurons rely on localized mitochondria positioned throughout their extended projections for a reliable supply of ATP that can be matched to spatiotemporally heterogeneous metabolic demand. Oxidative stress and aging lead to the decline of health in mitochondria situated in metabolic demand sites throughout the cell. This decline occurs on timescales of hours to days, while neuronal lifetimes are much longer. Since neuronal mitochondria have not been observed to replicate outside the soma, transport and delivery of fresh mitochondrial material from the cell body is necessary to maintain a healthy mitochondrial population at distal demand sites. Optimal distribution of healthy mitochondria in both distal and proximal high metabolic demand sites requires an interplay between the stationary mitochondria and a motile pool capable of bringing in new proteins from the soma. This transport-based interplay can occur via two mechanisms: transient fusion-fission events leading to equilibration of health or by intermittent switching between the stationary and motile pools (stop-and-go). We develop a quantitative model of mitochondrial maintenance via these mechanisms, delineating the key parameters that govern steady-state health of mitochondria situated at discrete regions throughout the length of a neuronal projection. Our analysis indicates that very infrequent exchange between the stationary and motile pools is optimal for healthy distal mitochondria. When the total number of mitochondria in a projection is fixed, both exchange mechanisms yield similar outcomes and can be optimized further by selective recycling of damaged mitochondria via mitophagy. However, the exchange of mitochondrial contents through transient fusion events is more robust than the stop-and-go mechanism, and can be further enhanced by mitophagy. These results provide a framework for quantifying how organelle transport and interactions affect mitostasis in neurons, a key to understanding many neurodegenerative diseases.

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Misregulation of mitochondria-lysosome contacts by GBA1 dysfunction in dopaminergic neuronal models of Parkinson's disease
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Mitochondria and lysosomes are both key cellular organelles which crosstalk with one another and have been implicated in multiple diseases including Parkinson's disease. Mitochondria-lysosome contacts were previously identified in several cell types, but their role in human neurons has not been investigated. Using super-resolution time-lapse imaging in iPSC-derived dopaminergic neurons, we
found that mitochondria-lysosome contacts dynamically form in the soma, axons, and dendrites of human neurons to mediate organelle crosstalk. To further investigate the dynamics of these contacts in disease, we examined GBA1-linked Parkinson’s disease (PD), as the lysosomal enzyme GBA1 represents the most common risk factor for Parkinson’s, and is linked to decreased activity of the lysosomal glucocerebrosidase protein and lysosomal dysfunction. Parkinson’s disease patient neurons harboring GBA1 mutations exhibited prolonged mitochondria-lysosome contacts due to defective modulation of the untethering protein TBC1D15, which was further linked to disrupted mitochondrial distribution and decreased ATP levels. Importantly, expression of TBC1D15 which promotes mitochondria-lysosome contact untethering was sufficient to rescue these phenotypes in Parkinson’s patient-derived dopaminergic neurons. Together, our work demonstrates an important role for mitochondria-lysosome contacts as an upstream regulator of mitochondrial function and dynamics in midbrain dopaminergic neurons, and as a potential contributor to the pathogenesis of Parkinson’s disease.

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**Mitochondrial motility scales with dendritic architecture in neurons in vivo**

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Mitochondria are dynamic organelles that grow and degrade, divide and fuse, and move along cytoskeletal filaments within cells. The molecular mechanisms underlying mitochondrial dynamics are well defined. However, it is unclear how these molecular mechanisms are integrated over several orders of magnitude to give rise to stable, cell-scale mitochondrial distributions. Maintaining steady-state mitochondrial distributions is particularly challenging in complex, elaborately branched neurons. In this work, we investigated the relationship between mitochondrial motility and dendritic architecture in vivo using well-characterized HS neurons in the *Drosophila* visual system. HS function relies on the integration of synaptic inputs across its highly branched dendritic arbor, with individual dendritic branches summing with approximately equal weight. HS dendritic branches should therefore have equivalent energetic demands. Consistent with this, by imaging GFP-tagged mitochondria in HS neurons in fixed brains we found that mitochondrial densities are roughly uniform from branch-to-branch. In addition, we measured significant mitochondrial motility throughout HS dendritic arbors in intact flies using in vivo confocal microscopy. In the primary dendrite, anterograde mitochondrial movement is balanced by retrograde movement, and average speeds range from 0.2-0.6μm/s in primary and distal dendrites. To determine how HS neurons maintain stable branch-to-branch mitochondrial densities despite high motility rates, we compared measurements of dendritic architecture with mitochondrial movement. Dendritic arbors can be decomposed into successive subtrees, and we reasoned that steady-state mitochondrial distributions could be maintained if, at each branch point, the relative amount of mitochondrial movement through each daughter branch scales with the relative size of the subtree supported by that branch. By measuring dendritic subtree lengths, we found, first, that HS dendritic branch patterns are highly asymmetric: one daughter subtree, is, on average, approximately 2.5 times longer than the other. Second, dendritic subtree length asymmetry is tightly correlated with the dendritic cross section area asymmetry: larger subtrees have proportionally thicker trunks. Finally, we found that mitochondrial transport also scales with dendrite thickness, with proportionally more mitochondria moving into the thicker daughter branch. Together, these results show that mitochondrial
Physiological Neuronal Stimuli Drive Diverse Calcium Signals in the Endoplasmic Reticulum *In Vivo*

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Intracellular Ca\(^{2+}\) regulates a multitude of neuronal processes, including synaptic plasticity and neurotransmission. The endoplasmic reticulum (ER) is a major Ca\(^{2+}\) reservoir in neurons. Although much is known about the molecular mechanisms underlying ER Ca\(^{2+}\) uptake and release, it is unclear how ER Ca\(^{2+}\) handling contributes to neuronal activity in vivo. In this work, we investigated the role of the ER in activity-driven intracellular Ca\(^{2+}\) signaling in the *Drosophila* visual system. In particular, we focused on the neurons that comprise local motion detection circuits. Within these circuits, changing light intensities drives cytosolic Ca\(^{2+}\) signals in constituent neurons, with different cell types exhibiting diverse response kinetics spanning tens of milliseconds to second timescales. This cell-type specific temporal filtering is critical for motion detection, but the cellular mechanisms underlying temporal filtering are unknown. Ca\(^{2+}\) handling by the ER can regulate cytosolic Ca\(^{2+}\) signals over a broad range of timescales, and we therefore hypothesized that the ER tunes cell-type specific temporal filtering in local motion circuits. To test this, we used *in vivo* two-photon microscopy to measure visual stimulus-evoked ER and cytosolic Ca\(^{2+}\) signals in neurons in intact flies. We found, first, that the same visual stimulus drives dramatically different Ca\(^{2+}\) signals in the ER depending on the neuronal cell type and compartment. For example, within one cell type (Mi1 neurons), distal dendrites exhibit fast, transient (~100ms) ER Ca\(^{2+}\) uptake, proximal dendrites exhibit slow, sustained (~1s) ER Ca\(^{2+}\) release, and axon terminals exhibit rapid, sustained (~100ms-1s) Ca\(^{2+}\) uptake. Thus, the ER can act as either a Ca\(^{2+}\) source or sink during neuronal activation over a range of timescales. Second, we found that cell-type specific RNAi knockdown of the ER Ca\(^{2+}\) efflux channel inositol trisphosphate receptor (IP\(_3\)R) perturbs activity-driven ER and cytosolic Ca\(^{2+}\) signals. Interestingly, IP\(_3\)R knockdown reduces the amplitude of ER Ca\(^{2+}\) uptake in Mi1 axons and distal dendrites as well as ER Ca\(^{2+}\) release in proximal dendrites, suggesting that homeostatic mechanisms act to reduce Ca\(^{2+}\) uptake when Ca\(^{2+}\) release is impaired. Finally, we found that reduced ER Ca\(^{2+}\) uptake in Mi1 axon terminals after IP\(_3\)R knockdown is associated with more sustained cytosolic Ca\(^{2+}\) responses to visual stimuli, suggesting that Ca\(^{2+}\) handling by the ER regulates the temporal filtering properties underlying motion detection. Altogether, we propose that the ER plays a critical role in regulating activity-driven intracellular Ca\(^{2+}\) signals in neurons *in vivo*, thereby tuning single-cell processing as well as large-scale neural circuit function.
regulation of mitochondrial trafficking and anchoring is therefore critical for neurons to meet enhanced energy requirements during sustained synaptic activity. However, underlying mechanisms sensing increased presynaptic ATP consumption remain elusive. Here, we reveal a new energy signaling axis that controls presynaptic mitochondria maintenance. Activity induces presynaptic energy deficit that could be rescued by recruiting mitochondria through energy sensing AMPK-PAK pathway. Synaptic activity induces AMPK activation within distal axonal compartments and AMPK-PAK signaling triggers phosphorylation of myosin VI, which drives presynaptic mitochondrial recruitment and syntaphilin-mediated anchoring on presynaptic F-actin. This pathway maintains presynaptic energy supply and calcium clearance during intensive synaptic activity. Disrupting this signaling cascade triggers local energy deficits and $[Ca^{2+}]_i$ buildup, leading to impaired synaptic efficacy and accelerated synaptic depression. Our study reveals a new mechanistic crosstalk between energy sensing and mitochondria trafficking/anchoring to maintain presynaptic metabolism, thus sustaining short-term synaptic plasticity and prolonged synaptic efficacy. (This work was supported by the Intramural Research Program of NINDS, NIH ZIA NS003029, and ZIA NS002946).

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Endoplasmic Reticulum morphological regulation and luminal transport- underlying mechanism and reliant neuronal cell functionalities

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The Endoplasmic Reticulum (ER), a network of membranous sheets and pipes, supports functions encompassing biogenesis of secretory proteins and delivery of functional solutes throughout the cell periphery. Molecular mobility through the ER network enables these functionalities. The diffusion-driven molecular motion (traditionally presumed by default), alone is not sufficient to explain the kinetics of luminal transport across supramicron distances. Understanding the ER structure-function relationship is critical to rationalising how mutations in ER morphogenic proteins give rise to neurodegenerations. A purpose-developed super-resolution microscopy and data analysis approach enabled us to deconvolute the molecular motion inside the ER and observe structural dynamics in greater detail: Stochastic analysis of single-particle trajectories of ER luminal proteins revealed that the topological organisation of the ER correlates with distinct trafficking modes of its luminal content: with a dominant diffusive component in tubular junctions and a fast flow component in tubules (Holcman et al. 2018 Nature Cell Biology). Particle trajectory orientations resolved over time revealed an alternating current of the ER contents. Ultra-fast structured illumination microscopy identified energy-dependent tubule contraction events at specific points as a plausible mechanism for generating active ER luminal flow (a membrane-curving process resulting in nanoperistalsis-like content propulsion). An AI-assisted photoactivation pulse-chase methodology, we have developed provides additional detail on the characteristics of the active ER transport. The discovery of active flow in the ER has implications for timely ER content distribution throughout the cell, particularly important for cells with expensive ER-containing projections, e.g. neurons, with potential repercussions of its perturbations on cell proteostasis and signalling. In particular, our in-silico modelling and experimental measurements of calcium dynamics in induced Pluripotent Stem cells-derived cortical neurons with manipulated ER, reveal how the ER morphoregulation and dynamics define (patho)physiological calcium signalling. The
identified ER perturbation-sensitive functional nodes and their relationship with ER stress will be also discussed.

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A retrograde transit filter removes mitochondria near distal nodes of Ranvier

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Maintaining organelle homeostasis is a major challenge for neurons, as most organelles have to be transported between sites of initial biogenesis and eventual removal. For instance, mitochondria are generated mainly in the soma and anterogradely transported into remote axonal branches and synapses, where neurotransmission critically depends on energy supply. At the same time, many mitochondria are shuttled back to the soma by retrograde transport. Still, a substantial excess of mitochondria is exported into axons than is later retrieved—raising the question of how mitochondrial homeostasis (‘mitostasis’) is normally achieved and what mechanisms underlie mitostatic dysregulation e.g. in Parkinson’s or motor neuron disease. Based on measurements of mitochondrial mass balance in mouse motor axons, we identified a major site of degradation near synapses. Using pulse-chase tracking of photo-tagged synaptic mitochondria in Thy1-mitoDendra mice, we discovered that ~60% of the mitochondria that attempted to exit neuromuscular synapses stopped and disappeared just before the most distal node of Ranvier. Further capture events occurred with decreasing frequency at more proximal nodes. In line with local degradation, we found a high density of mitolysosomes at such ‘capture’ sites. Moreover, when synaptic mitochondria were depolarized locally using a photo-caged uncoupler, capture rates increased. Accordingly, in mouse models of motor neuron disease, where synaptic mitochondria accumulate damage, we observed increased capture. Our ongoing genetic analysis shows that ablation of Pink1/Parkin-mediated mitophagy resulted in increased capture and reduced retrograde transport of mitochondria, suggesting that Pink1/Parkin act in parallel or downstream of the quality control system that we identified. In summary, our data support a new model, where mitochondrial quality control takes place in distal axons and synapses. Perinodal sites act as a cascade of axonal checkpoints to filter out and degrade damaged mitochondria as they transit towards the soma—a mitostatic system that establishes mitochondrial mass balance in healthy axons and is disrupted in degenerative axonopathies.

Physical Approaches to Cell Biology 1

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Estimation of material properties of cellular organelles from active shape fluctuations in the living cells

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The material properties of organelles meet their biological functions. However, an understanding of cellular functions from the mechanical point of view is largely lacking due to limitation of experimental methods. Here, we measure the material properties of cellular organelles and force generation in the living cells from their active shape fluctuations such as filaments and vesicles. The shapes of cellular organelles are actively fluctuating in the living cells and their fluctuations are determined by force balance between active forces, viscoelasticity of the cytoplasm, and the material properties of cellular organelles. Combining the theory with microrheological measurement of the cytoplasm with magnetic tweezers, we estimate elasticity of various organelles and force generation inside the cell.

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**Regulation of Three-Dimensional Epithelial Cell Shape in a Two-Dimensional Tissue**

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Three-dimensional force distribution within the actin cytoskeleton of epithelial tissue regulates cell shape. While two-dimensional cell shape has been well characterized and heavily studied, three-dimensional cell shape regulation is less well understood despite its critical role in large scale epithelial processes such as invagination. By examining the relationship between cell height, density and biological components of the actin cytoskeleton, we explore the mechanisms by which epithelial cells regulate shape and volume. We observe that while cell density is not a strong indicator of epithelial height, osmotic shock drastically decreases both tissue height and cell volume while leaving the lateral shape of cells in the tissue undisturbed. In addition, we examine the relationship between monolayer maturity and cell volume variation and neighbor correlation of individual cell volume and height.

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**DNA origami demonstrate the unique stimulatory power of single pMHCs as T-cell antigens**

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T-cells detect with their T-cell antigen receptors (TCRs) the presence of rare agonist peptide/MHC complexes (pMHCs) on the surface of antigen presenting cells (APCs). How extracellular ligand binding triggers intracellular signaling is poorly understood, yet spatial antigen arrangement on the APC surface has been suggested a critical factor. To examine this, we engineered a biomimetic interface based on laterally mobile functionalized DNA origami platforms, which allow for nanoscale control over ligand distances without interfering with the cell-intrinsic dynamics of receptor clustering. When targeting TCRs via stably binding monovalent antibody fragments we found the minimum signaling unit promoting efficient T-cell activation to consist of two antibody-ligated TCRs within a distance of 20 nanometers. In contrast, transiently engaging antigenic pMHCs stimulated T-cells robustly as well-isolated entities. These results identify pairs of triggered TCRs as minimal receptor entities for initiation of signaling, yet validate the exceptional stimulatory potency of isolated single pMHC molecules.
**P97**

**Tracking the generation of Copy Number Variation by live cell imaging**

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Copy number variation (CNV) is common in most cancers, especially solid tumors [1]. To visualize the development of CNV, we have been doing live cell imaging of a lung cancer line with an RFP tag fused to one gene on one allele of one chromosome. As the cell cycle progresses, RFP intensity of the tagged protein normally increases, and peaks at mitosis - whereas some cells lose the RFP protein at division and not only fail to show such an increase before the next mitosis but become more dim. Prolonged mitosis induced by inhibition of the spindle assembly checkpoint (SAC) tends to increase such events. We also observed micronuclei generation during such transitions, consistent with a mechanism for chromosome mis-segregation into micronuclei during CNV generation. While such observations confirm the viability of the engineered cells and suggest a heritable genetic change, we confirmed the approach as a reporter for live cell chromosome loss by multiple genetic analyses. Single cell DNA sequencing is the most exacting method and confirms that the RFP-negative cells lose the entire, edited chromosome. The reporter thus provides a powerful approach to study sources of the genetic variation that is a hallmark of cancer. [1] Pfeifer CR, et al. Genome variation across cancers scales with tissue stiffness - an invasion-mutation mechanism and implications for immune cell infiltration. *Curr Opin Syst Biol.* 2017.

**P98**

**Passive coupling of membrane tension with cell volume changes is actively regulated by mTORC2**

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Membrane tension orchestrates the dynamics of cellular membrane functions. In physiologically-relevant conditions, cells experience dramatic changes of their osmotic environment, strongly affecting membrane tension, cell volume and related cellular functions. Numerous processes - from synthesis of osmoprotectant molecules to ion pumping - control cell volume and membrane tension changes, participating in the protective cell response ensuing from osmotic shocks. However, a quantitative characterization of membrane tension and cell volume changes after osmotic shocks is lacking. Here, we performed time resolved membrane tension and cell volume measurements during osmotic shocks using tube pulling technique and Flipper-TR probe: a novel probe to measure membrane tension. The cell response during a few seconds following the shock is purely passive: tension follows the volume change elastically, which varies to equilibrate osmotic pressures inside and outside the cell. At longer times, the response is highly asymmetric, with cells recovering from hypoosmotic shocks within tens of seconds, and not from hyperosmotic shocks for more than ten minutes. But during the entire response, membrane tension and cell volume changes are strongly coupled. Furthermore, pharmacological inhibition of ion pumping & tubulin polymerization strongly interfered with the hypoosmotic response and not with the hypertonic one, but tension and volume dynamics were still strongly coupled. Only when mTORC1&2 and actin were pharmacologically inhibited, the tension response was decoupled from
volume changes. These results argue for an active regulation by mTORCs of the passive coupling between membrane tension and cell volume changes during the osmotic response.

P99

**Scaling mechanical response and proliferation rate with cell size using apical stress fibers.**  
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The scaling of the properties of biological systems with their size is central to development and physiology. However, such scaling remains poorly explored in cell mechanics and mechanosensing. By examining how a Drosophila epithelium responds to morphogenetic forces, we identified a class of apical stress fibers (aSFs) anchored to adherens junctions. aSF number scales with cell apical area, in agreement with mathematical models showing that such scaling can prevent larger cells from elongating under mechanical stress. Furthermore, aSFs promote clustering of Hippo pathway components, thereby scaling Hippo activity and thus proliferation rate with area. We found that aSFs nucleate at tricellular junctions (TCJs), then move across the cell before eventually breaking, most often when they encounter another TCJ. This observation motivates a simple geometric mechanism that can quantitatively account for most of the scaling of aSF number with cell size: Because larger cells have more TCJs, they nucleate more aSFs; because these TCJs are typically farther apart, each aSF survives longer before breaking. Development, homeostasis, and repair entail changes in epithelial cell area driven by mechanical forces; our work highlights how, in turn, cell mechanosensitivity scales with cell area.

P100

**Microfluidic Guillotine Reveals Multiple Timescales and Mechanical Modes of Wound Response in Stentor coeruleus**  
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Wound healing is a key feature of living systems. At the single cell level, the ability to heal wounds underpins fundamental biological processes from cytokinesis to cancer metastasis. For some organisms, wound healing can involve short-term sealing of the plasma membrane opening and long-term regeneration to rebuild damaged or lost structures. However, many open questions remain on the mechanisms and dynamics of single-cell wound healing. Here we describe a microfluidic “guillotine” as a platform to probe the mechanisms and timescales of single-cell wound healing using Stentor coeruleus, a giant ciliate, as our model. In this study, cells were flowed by a syringe pump and bisected by a microfluidic guillotine, which is a knife blade fabricated inside a microfluidic channel. Varying the flow rate yields two wounding regimes of the guillotine: a low viscous stress Regime 1 and a high viscous stress Regime 2. To quantify the time of wound repair, we fixed cells at selected time points after wounding and stained them with Sytox Green, a membrane impermeable dye. Regime 1 wounds are repaired on the order of 10² s while the more severe Regime 2 wounds are repaired on the order of 10³ s. The survival rate derived from the Sytox Green assay agrees with the survival rate of cells observed over 24 hours. Further, we observed three mechanical modes of wound response in Stentor: (1) contraction where the cell folded around the wound site typically over a 100-250 s period, (2) cytoplasm
retrieval where extruded cytoplasm was pulled back into the cell body over 20-200 s, and (3) swimming where the cell used a swimming motion to detach from extruded cytoplasm over 20-100 s. Cilia are important for the swimming mode, as cilia inhibition by NiCl₂ significantly increased the time for the swimming mode to complete and reduced how frequently the swimming occurred. Surprisingly, myonemes, the contractile fibers in *Stentor*, were not important for any mode, as myoneme inhibition by KI did not affect the time for the contraction mode to complete and slightly increased how frequently the contraction occurred. While the precise mechanisms and pathways involved in each of these mechanical modes of wound repair remain unknown, we expect our wound healing platform, combined with tools such as RNAi, can create opportunities to further study the mechanisms of single-cell wound healing.

P101

Hydrodynamic Cell Splitter as a Microfluidic Tool to Study Single-cell Wound Healing and Regeneration

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The ability of living organisms to heal wounds and regenerate lost components is essential for maintaining homeostasis. At the cellular level, wound healing is initiated when the cell detects that the plasma membrane is compromised. Wound detection triggers cellular mechanisms which seal the membrane and consequently there is partial or full regeneration of organelles lost to wounding. Recently, a microfluidic guillotine was developed as a tool for bisecting cells repeatedly at the edge of a knife geometry at a high throughput of ~64 cells/minute with single celled *Stentor coeruleus* as the model organism. The operation of the microfluidic guillotine is limited by build-up of cellular debris at the knife tip during the cutting process. Here, we describe a hydrodynamic cell splitter for splitting cells using *Stentor coeruleus*, a single cell ciliate protist, as our model. The microfluidic device consists of a cross junction and cells entering the cross junction are split by the extensional fluid flow at a throughput as high as ~500 cells/min, nearly 8 times higher than the microfluidic guillotine. As the hydrodynamic cell splitter splits cells in a non-contact manner, the process is cleaner and there is no build-up of cellular debris. The splitting efficiency of the device, quantified as the percentage of cells split at the junction, increases with Reynolds number. At a Reynolds number of 362, the splitting efficiency is 97%. The survival rate quantifies the viability of the cells post-splitting and is measured as the percentage of the number of cell fragments generated during the experiment that are alive at 24 hours post-splitting. In the regime of high Reynolds numbers, the survival rate decreases with increasing Reynolds number, with a survival rate of around 70% at a Reynolds number of 362. This is ~10% higher than regime II cuts in the microfluidic guillotine. Our model organism, *Stentor coeruleus*, exhibits robust wound repair and regeneration capabilities. The viability of the wounded cell depends on two competing timescales associated with (a) wound closure and (b) the spilling of cellular contents out through the wound site. Therefore, the size of the cell and the wound are important parameters determining the kinetics of wound repair and regeneration. However, it is unknown if there exists a limit on the size of the wounded cell fragment capable of fully regenerating. By scaling down the hydrodynamic splitter and serially splitting *Stentor* cells to generate specific cell fragment sizes, we expect to characterize the smallest cell fragment capable of regenerating. This would be essential in pinpointing relevant cell components associated with the regeneration process and exploring the notion of cell memory which enables cells to recreate lost cell anatomy.
**P102**

**Stretching DNA Origami: Effect of Nicks and Holliday Junctions on the Axial Stiffness**

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A hallmark of DNA nanotechnology is the ability to assemble complex nanostructures with precision that can rival the complexity found in natural macromolecular assemblies. Therefore, DNA nanotechnology has found numerous applications. For example, DNA origami-based drug delivery vehicles are subject to hydrodynamic forces induced by patients’ circulation. Such vehicles should be designed to withstand the hydrodynamic forces without prematurely leaking the drugs before reaching the destination. In fact, the design rules to accomplish prescribed mechanical properties during assembly are crucial for all applications where the DNA nanostructures are subjected to external loads. Adequate stiffness of DNA hydrogels, as tissue engineering scaffolds, is desirable to regulate cellular functions; precise elasticity of DNA tension sensors has to be achieved to measure contractile forces generated by single cells. However, the impact of the design features on the resulting structural integrity and functionality of the nanostructures remains largely understudied. To address this knowledge gap, we have systematically studied axial stiffness of DNA nanostructures as a function of key design motifs by combining experiments and modeling. Four constructs of two-helix nanobeams with specified densities of nicks and Holliday junctions are synthesized and stretched by fluid flow. Implementing single particle tracking to extract force-displacement curves enables the measurement of DNA origami stiffness values at the enthalpic elasticity regime. Comparisons between ligated and nicked helices show that the latter exhibit nearly a two-fold decrease in axial stiffness. Numerical models that treat the DNA helices as elastic rods are used to evaluate the local loss of stiffness at the locations of nicks and Holliday junctions. It is shown that the models reproduce the experimental data accurately, indicating that both of these design characteristics yield a local stiffness two orders of magnitude smaller than the corresponding value of the intact double-helix. This local degradation in turn leads to a macroscopic loss of stiffness that is evaluated numerically for multi-helix DNA bundles. Counterintuitively and strikingly, our numerical models indicate that a very large number (>16) of helices would be required to be included in the assembly to recover the stiffness of intact dsDNA. Our results are useful for the design of many DNA-based materials and provide rules to reduce over-engineering, minimize unnecessary complexity and thus enable the synthesis of robust higher-order DNA nanostructures.

**P103**

**Mass measurements during lymphocytic leukemia cell polyploidization decouple cell cycle and cell size dependent growth**


Cell size is believed to influence cell growth and metabolism. Consistently, several studies have revealed that large cells have lower mass accumulation rates per unit mass (i.e. growth efficiency) than intermediate sized cells in the same population. Size-dependent growth is commonly attributed to
transport limitations, such as increased diffusion timescales and decreased surface-to-volume ratio. However, separating cell size and cell cycle-dependent growth is challenging. To address this, we monitored growth efficiency of pseudodiploid mouse lymphocytic leukemia cells during normal proliferation and polyploidization. This was enabled by the development of large-channel suspended microchannel resonators that allow us to monitor buoyant mass of single cells ranging from 40 pg (small pseudodiploid cell) to over 4000 pg, with a resolution ranging from ~1% to ~0.05%. We find that cell growth efficiency increases, plateaus, and then decreases as cell cycle proceeds. This growth behavior repeats with every endomitotic cycle as cells grow in to polyploidy. Overall, growth efficiency changes 33% throughout the cell cycle. In contrast, increasing cell mass by over 100-fold during polyploidization did not change growth efficiency, indicating exponential growth. Consistently, growth efficiency remained constant when cell cycle was arrested in G2. Thus, cell cycle is a primary determinant of growth efficiency. As growth remains exponential over large size scales, our work finds no evidence for transport limitations that would decrease growth efficiency.

Receptors and Regulation

P104

Genetic and proteomic screens reveal a connection between mechanosensitive ion channel MSL10 and ER-plasma membrane contact sites

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It has recently become clear that many parts of a cell are involved in the perception and response to mechanical forces. Cellular membranes can be tethered to each other at locations called membrane contact sites, and these are important locations of signaling, ionic flux, and lipid transfer in both plants and animals. It is unknown whether membrane contact sites can function in the perception of and response to mechanical information. Here, we show that a mechanosensitive ion channel from *Arabidopsis thaliana*, MSL10, genetically and physically interacts with proteins at endoplasmic reticulum-plasma membrane (ER-PM) contact sites. We have previously shown that MSL10 transduces multiple responses to osmotic cell swelling, and the objective of this study was to identify components of its signaling pathway that lead from cell swelling to programmed cell death. I found that MSL10 co-immunoprecipitates with the vesicle-associated proteins VAP27-1 and VAP27-3 and synaptotagmin (SYT)1, major constituents of ER-PM contact sites in plants. MSL10 and its orthologs have a non-canonical FFAT motif for binding to VAPs, which also contains an important residue, Ser640, which when mutated to Leu constitutively promotes cell death. Furthermore, a forward genetic screen for genetic suppressors of the gain-of-function *MSL10*<sup>S640L</sup> (*msl10-3G* allele) identified missense mutations in *SYT5* and *SYT7*. At ER-PM contact sites, SYT5 and SYT7 are known to interact with SYT1, which helps maintain PM integrity in response to mechanical pressure. Taken together, these results suggest that the cell death-promoting activity of MSL10 can be modulated by its association with ER-PM contact site proteins, and raises the possibility that MSL10 regulates ER-PM connectivity. We hypothesize that MSL10 transduces mechanical information from cell swelling to promote a loss of ER-PM contacts through decreased VAP binding, which can be rescued by increased activity of synaptotagmins.
A reverse genetics-based deubiquitinase (DUB) screen for receptor tyrosine kinase stability & turnover

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Receptor tyrosine kinases (RTKs) are important regulators of signal transduction, cell function and animal physiology. RTKs frequently undergo ubiquitination/deubiquitination cycles which regulate membrane trafficking, proteolysis, signaling and cell function. One such RTK is vascular endothelial growth factor receptor 2 (VEGFR2), which regulates endothelial cell function and angiogenesis i.e. sprouting of new blood vessels from pre-existing ones. Angiogenesis is disrupted in pathological conditions such as cancer. Targeting tumor angiogenesis is thus highly relevant for cancer therapy. A number of studies suggest that VEGFR2 undergoes basal and VEGF-regulated ubiquitination, which impacts on VEGFR2 turnover and downstream signaling. We have identified the deubiquitinase USP8 as a candidate DUB that regulates VEGFR2 trafficking and turnover. However, there is likely to be redundancy and complexity in VEGFR2 deubiquitination due to there being ~110 DUB family gene products. Our objective was therefore to perform an unbiased screen of all known DUBs. In this study, we used a reverse genetics approach of RNAi-based screening to screen the human DUB family for influence on VEGFR2 levels in primary human endothelial cells. A high throughput microscopy-based assay was used to screen DUB gene products. DUB knockdown using siRNA produced three observable VEGFR2 phenotypes: VEGFR2 levels were unchanged, elevated or decreased. We also evaluated effects of DUB depletion in either the absence (basal) or presence (ligand-stimulated) of VEGF-A, a potent growth factor that regulates endothelial cell function and angiogenesis. Comparison of the DUB families suggest that different DUB family members might have markedly distinct roles in either directly acting on VEGFR2, or indirectly regulating factors linked to VEGFR2 trafficking and/or turnover. We suggest models to reconcile the data from our DUB screen on VEGFR2 signaling, trafficking, turnover and endothelial cell function.

Assessing the role of Y265 phosphorylation on Cx43 internalization

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Misregulation of the gap junction protein Connexin 43 (Cx) due to mutations and altered phosphorylation events is known to be related to cardiac disease, hearing loss, cancers and vision problems. Cx43 displays a remarkably short half-life for a membrane protein, of approximately 1-5 hours. Internalization of Cx43 is achieved by clathrin-mediated AP2 binding on the C-terminus. The C-terminus is critical in the regulation of Cx43 internalization and gap junction communication. In the past, our work has been focused on characterizing the relationships between Serine phosphorylation events and internalization. However, the role of tyrosine phosphorylation on internalization remains less understood. In order to assess the importance of tyrosine phosphorylation in Cx43 internalization, we mutated Y247 and Y265 residues of Cx43 and transiently transfected it in cell lines. These residues are phosphorylated by v-Src. Our experiments show that preventing phosphorylation of Y265 leads to
increased gap junction plaque size compared to wild type Cx43. Phosphorylation of Y265 is correlated with the opening of gap junction channels and increased communication. Interestingly, our data suggests that Y265 phosphorylation is dependent on S368 phosphorylation, a signal for gap junction closure and internalization. Additionally, we expect to see longer half lives when tyrosine phosphorylation is prevented. The impact of phosphorylation on AP-2 will be investigated as well, considering that Y265 interacts directly with AP-2, a major step in Cx43 internalization. We will generate Cx43 tyrosine mutants with a GFP tag and investigate in real time the effect of phosphorylation of Y265 and Y247 in the internalization process. We will also examine if there are compensating phosphorylation events to trigger internalization when phosphorylation on the tyrosine residues is prevented.

P107

Development of an ELISA for zebrafish leptinA
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LeptinA is a 16kDa protein that regulates food consumption, metabolic rate, and bone mass in zebrafish. The most common way to test leptinA expression is through Western Blot, which requires several micrograms of tissue for accurate detection. Because of this, some zebrafish tissues can only be tested by pooling samples. An Enzyme-Linked Immunosorbent Assay (ELISA) can detect concentrations of leptinA in the picogram concentration using less tissue than needed for a Western Blot. Until now, such an ELISA has not been developed for zebrafish. To verify ELISA sensitivity, we created a quantitative standard curve in Excel using serial dilutions ranging 1:1 to 1:32 of concentrated leptinA diluted in 50mM Tris. ELISA recovery sensitivity was tested by spiking serum samples with known concentrations of leptinA and calculating recovery percentage. ELISA specificity was tested by running samples of proteins with similar molecular weight (ghrelin, growth hormone) and against bovine serum albumin. Here we demonstrate that wild type zebrafish leptinA levels are ~25.36 ng leptin per 1 ug of protein in the brain, ~17.05 ng of leptin per 1 ug of protein in muscle, and ~15.79 ng leptinA per 1 ug of protein in heart tissue. We developed this protocol for easier testing of individual zebrafish tissues and to test circulating leptinA levels in zebrafish for the first time.

P108

Endothelial PAR1-specific deubiquitinases regulates p38 inflammatory signaling
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Endothelial dysfunction is a hallmark of vascular disease and induced by various inflammatory mediators which signal through GPCRs pathways that are poorly understood. Thrombin induces endothelial inflammatory responses through the activation of protease-activated receptor 1 (PAR1), a GPCR. Previously, we showed that thrombin-activated PAR1 is modified by ubiquitin and drives recruitment of transforming growth factor-β-activated kinase-1-binding protein 2 (TAB2), an adaptor protein that binds TAB1 to trigger p38-dependent endothelial barrier disruption, a hallmark of inflammation. However, the regulatory processes that control PAR1-deubiquitination and associated p38 inflammatory signaling are not known. To fill this gap in knowledge, we sought to identify PAR1-specific deubiquitinases (DUBs) that removes K63-linked ubiquitin by conducting an unbiased comprehensive genome-wide siRNA library screen targeting all 96 human DUB genes in human cultured endothelial cells using multiple PAR1
ubiquitin-dependent functional readouts. We hypothesize that a PAR1-specific DUB is essential for terminating thrombin-induced p38 inflammatory signaling and might be altered in endothelial dysfunction. In an initial screen, endothelial cells were transfected with siRNA pools targeting specific DUBs and PAR1-ubiquitin dependent responses were assessed. We specifically examined PAR1 cell surface expression, controlled by basal PAR1 ubiquitination, and thrombin-stimulated p38 activation and downstream inflammatory signaling, which is dependent on agonist-triggered PAR1 ubiquitination. In preliminary studies have identified several candidates PAR1-specific DUBs. Currently, we are now investigating the function of top candidate DUBs in regulating the status of PAR1 ubiquitination, cell surface expression and p38 inflammatory signaling. The molecular mechanism responsible for PAR1 deubiquitination and relation to controlling the spatio-temporal dynamics of pro-inflammatory signaling will be further investigated. Taken together, we anticipate that these studies using a comprehensive siRNA library screening approach will reveal the identity of several DUBs that have important functions in regulating PAR1-induced ubiquitin driven p38 MAPK endothelial inflammatory signaling.

Rho-family GTPases

P109

3D visualization of small GTPase activity during megakaryocyte differentiation and platelet formation

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Our primary objective is to elucidate the spatio-temporal dynamics of Ras- and Rho-family small GTPase activity underlying proplatelet formation. Megakaryocytes (MK) are specialized cells responsible for platelet production in the human body. During a process called proplatelet formation (PPF), MKs undergo a series of poorly understood and dramatic morphological changes to transform from large round cells (diameter 50-100 µm) into dynamic, complex structures containing many long bifurcated protrusions. GTPases likely regulate these astonishing morphological rearrangements during platelet production, but little is known about their spatio-temporal dynamics or coordination. Using a combination of Lattice Light Sheet Microscopy (LLSM) and FRET-based biosensors, we captured the complex 3D nature of PPF while quantifying GTPases activity. Long-term imaging of MKs undergoing differentiation revealed an intricate dance between extension and organization of small structures (proplatelets) within protrusions. We quantified the activity of Rap1, Cdc42, and RhoA GTPases, revealing microregions of activity throughout the platelet process preceding the extension and organization of protrusions. This study shines a light into transient localized signaling events, and how they generate the morphological changes required for PPF. The work could ultimately help create strategies to overcome the limited platelet production in vitro.
Optogenetic control of small GTPases reveals RhoA-mediated intracellular calcium signaling
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Rho/Ras family small GTPases are known to regulate numerous cellular processes, including cytoskeletal reorganization, cell proliferation, and cell differentiation. These processes are also controlled by Ca\textsuperscript{2+}, and consequently, crosstalk between these signals is considered likely. However, systematic quantitative evaluation is not yet reported. Thus, we developed optogenetic tools to control the activity of small GTPases (RhoA, Rac1, Cdc42, Ras, Rap, and Ral) using an improved light-inducible dimer system (iLID). Using these optogenetic tools, we investigated calcium mobilization immediately after small GTPase activation. Unexpectedly, we found that only RhoA activation induced a transient intracellular calcium elevation in RPE1 and HeLa cells. Transients were also observed in MDCK and HEK293T cells by RhoA activation, but interestingly, molecular mechanisms were identified to be different among cell types. In RPE1 and HeLa cells, RhoA directly activated phospholipase(PLC)ε at the plasma membrane, which in turn induced Ca\textsuperscript{2+} release from the endoplasmic reticulum (ER). The RhoA-PLCε axis induced calcium-dependent NFAT nuclear translocation, suggesting it does activate intracellular calcium signaling.

Investigating Anillin’s Role in Coordinating Contractility at Epithelial Cell-Cell Junctions
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Epithelial cells play an important role in the development and health of an organism by generating specialized barriers. In order to form a functional barrier, epithelial cells adhere to one another through cell-cell junctions. Cells in epithelial tissues are subject to various cell shape change events, ranging from cell-scale events like cell division to tissue-scale rearrangements during morphogenesis. Cell-cell junctions are dynamic, adaptive structures that must be continually remodeled during these epithelial cell shape changes, while still maintaining a functional barrier. However, the mechanisms underlying junction maintenance, repair, and remodeling are not well understood. Our lab has shown that one mechanism underlying tight junction repair involves Rho flares, short-lived local accumulations of active Rho, which trigger actomyosin contractility to repair tight junctions. Anillin, a scaffolding protein that is well known for its role during cytokinesis, has also been shown to regulate cell-cell junctions. Anillin organizes both junctional and medial-apical actomyosin and promotes junctional contractility by increasing the membrane residence time of active Rho, giving it increased access to effectors. Interestingly, Anillin was also reported to negatively regulate Rho in a tension-dependent manner during cytokinesis by recruiting p190RhoGAP-A. We found that Anillin and p190 colocalize at Rho flares, leading us to hypothesize that Anillin coordinates contractility at Rho flares by extending Rho membrane residence time to support contractility, and, once sufficient contractility has been generated, recruiting p190 to limit excess contractility. In order to investigate Anillin’s role in regulating contractility a Rho flares, we knocked down Anillin in the Xenopus embryonic epithelium and measured the effects on Rho flares. Our preliminary data demonstrates that Anillin knockdown increased the incidence of flares, yet shortened their duration. In ongoing experiments, using a live imaging barrier assay developed in our lab (ZnUMBA), we will quantify the functional effect of Anillin knockdown barrier breaches and Rho flare-driven tight junction repair. Additionally, we will knock down p190 and measure how Rho flares and
barrier function are affected when p190 is perturbed. These results will strengthen our understanding of the mechanisms that regulate junction maintenance and repair and shed light on emerging roles of Anillin at epithelial cell-cell junctions.

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**Lights, Camera, Action: Investigating the mechanism of Rho flare activation using optogenetics**

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**Lights, Camera, Action: Investigating the mechanism of Rho flare activation using optogenetics**
Shahana A. Chumki1, Rachel E. Stephenson2, and Ann L. Miller1,2 Cell and Molecular Biology Graduate Program1, and Department of Molecular, Cellular and Developmental Biology2, University of Michigan, Ann Arbor, MI. Epithelial tissues generate barriers that compartmentalize multicellular organisms, support homeostatic nutrient and water transport, and prevent pathogens from invading the tissue. Epithelial cell-cell junctions, including tight junctions (TJs), which seal the paracellular space between cells, and adherens junctions, which promote cell-cell adhesion, are connected to an apical bundle of F-actin and Myosin II. Cell shape changes driven by dynamic events including cell division, wound healing, and developmental morphogenesis generate tensile stress on junctions and challenge cell adhesion and barrier integrity. We discovered that the small GTPase RhoA is activated at junctions in short-lived, local accumulations, termed “Rho flares”, which repair local leaks in barrier function via an actomyosin-dependent contraction of the junction. However, the mechanism by which Rho flares are activated at cell-cell junctions remains elusive. Because Rho flares are preceded by junction elongation and a local loss of TJ proteins, we predict that junction elongation may act as a mechanical stimulus that initiates tight junction leaks and the Rho flare repair pathway. Given the stochastic nature of Rho flares in the developing epithelium, I have adapted the previously described TULIP optogenetic system, which locally activates contractility upon light stimulation, for use in live *Xenopus laevis* embryos. I have shown that global light stimulation elicits recruitment of a photo-recruitable GEF followed by RhoA activation at epithelial cell-cell junctions in *Xenopus* embryos. By using regional light stimulation, I can induce regional epithelial contractility, which leads to an increased frequency of Rho flares at cell-cell junctions located nearby the region of stimulation. Using this approach, I am analyzing whether junction elongation occurs upstream of local TJ leaks and investigating the mechanism of Rho flare activation by GEFs. Our work highlights the versatility of the TULIP optogenetic system to induce contractility on demand to uncover the molecular players in this rapid TJ repair pathway.

P113

**Reconstitution of Excitable and Oscillatory Rho Waves**

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Cell-free reconstitution of biological systems is an essential tool for uncovering the molecular mechanisms that underlie cytoskeletal function. Here we describe the development of an ex vivo system that reconstitutes cellular cortical excitability on a supported lipid bilayer. In cells, cortical excitability is a phenomenon characterized by waves of active Rho (Rho-GTP) and actin polymerization that are
thought to “prime” cells to initiate actomyosin-dependent events such as cytokinesis and cell migration. Our group has previously characterized cortical excitability in developing *Xenopus laevis* embryos and starfish oocytes. We found that cortical waves are amplified by overexpression of the RhoGEF Ect2 and inhibited by actin filaments (F-actin). These results suggest that cortical excitability exists as an activator-inhibitor system, where both positive (Ect2-dependent) and negative (F-actin-dependent) feedback loops exist simultaneously to induce wave formation and propagation. We have now generated an *ex vivo* system using actin-intact *Xenopus* egg extract and a supported lipid bilayer that successfully reconstitutes both excitable and oscillatory active Rho waves. Upon addition to a lipid bilayer, egg extract generates a robust, excitable wave of Rho-GTP followed by actin polymerization. This first excitable wave has a phase shift between the Rho-GTP and F-actin that is comparable to *in vivo* cortical waves. This wave is quickly followed by the formation of an increasingly dense F-actin network and oscillatory active Rho waves. The periodicity of these oscillatory waves changes over time, which is in contrast to the stable period that has been characterized in cells. Both excitable and oscillatory waves are mediated by Rho signaling, as the addition of C3 transferase, a Rho inhibitor, blocks the formation of any *ex vivo* wave patterning. Reconstituted oscillatory Rho-GTP waves are also highly sensitive to the presence of F-actin. Surprisingly, the addition of the actin depolymerizer Latrunculin induces the rapid termination of oscillatory Rho-GTP patterning on the bilayer, whereas *in vivo*, Latrunculin addition amplifies Rho-GTP patterning. The addition of select factors that destabilize actomyosin (including SMIFH2, H-1152, SwinholiA, and Cofilin) also induces the rapid termination of oscillatory Rho-GTP patterning on the bilayer. Differences between reconstituted active Rho waves and cellular cortical waves offer a unique opportunity to investigate the various ways in which Rho-GTP and cytoskeletal patterning are regulated.

**P114**

**Ect2 and MPGAP drive the cortical excitability circuit**

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During cytokinesis in amphibian and echinoderm embryos, the cell cortex becomes excitable and spontaneously generates propagating waves of Rho activation and F-actin polymerization. This behavior, termed “cortical excitability”, operates under a reaction-diffusion model where Rho autoactivation hinges upon the Rho Guanine Nucleotide Exchange Factor (RhoGEF) Ect2, and Rho autoinhibition is mediated by a delayed, F-actin-dependent, feedback-loop. We report that M-Phase GAP (MPGAP/RGA3-4/ArhGAP11a), a Rho GTPase Activating Protein (RhoGAP), appears to play a role in regulating cortical excitability in both frogs and starfish. MPGAP displays excitable dynamics in both model systems and participates in Rho autoinhibition. Further, immature *Xenopus laevis* oocytes—which do not normally display cortical excitability—can be shifted into a high-amplitude, highly coherent, wave regime upon co-expression of both Ect2 and MPGAP, but not either protein on its own. Together, these findings support the model that balanced GEF and GAP activity is important for robust cortical excitability, and that Ect2 and MPGAP are likely major drivers of the system.
Second Messenger Systems in Signaling

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Local network of second messengers shaping the connectivity of the visual system.

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The connectivity between neurons is finely tuned in the visual system. Retinal ganglion cells (RGCs) form tightly organized connection maps in its two main targets, the superior colliculus (SC) and the dorsal-lateral geniculate nucleus (dLGN). These connections are formed during early stages of development. Second messengers, including cyclic nucleotides (cGMP and cAMP) and calcium are critical modulators of the development of neuronal connectivity. They are crucial for axon guidance and integrate extracellular signals received by growing axons. They are also involved in a wide range of cellular processes distinct from axon guidance. How they achieve specificity for each of their downstream effectors remain elusive. The diversity of these processes suggests that second messenger signals are tightly controlled in time and space to achieve the specific modulation of their downstream pathways. We aim to identify the spatio-temporal features of second messenger signals shaping nervous system connectivity. To be able to describe second messenger signals in different subcellular compartment and how they interact with one another we took advantage of an innovative toolbox: genetically encoded FRET based biosensors to detect and visualize second messenger signals and molecular sponges to induce subtle changes in second messenger concentration. Using SpiCee and SponGee, two molecular scavengers of calcium and cGMP respectively, we blocked second messenger signals in RGCs growth cones exposed to two repulsive molecules, ephrinA5 or Slit1. These cues are crucial for axon pathfinding at the optic chiasm and in the SC. We demonstrate that blocking these second messengers inside but not outside lipid rafts is sufficient to impair axonal response to ephrinA5. Two FRET-based biosensors (tHPDE5vv and Twitch2B) enabled to characterize the spatio-temporal features of cGMP and calcium signals in RGCs growth cones exposed to Slit1 or ephrinA5. We electroporated SpiCee and SponGee in embryonic retinas in utero, to alter calcium and cGMP signaling in RGCs in vivo. Our preliminary data suggest that terminal arbors of RGCs axons in the SC are altered by these manipulations, demonstrating a role of these two second messengers in axon guidance in vivo. Our observations highlight the crucial role of lipid rafts in the modulation of second messengers signals involved in axon guidance.

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The role of astrocyte store-operated Ca²⁺ entry in central sensitization following nerve injury in Drosophila

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Chronic pain following injury is a debilitating condition associated with serious comorbidities and opioid dependence. Understanding the neurological mechanisms that underlie chronification of pain is essential for prevention and treatment. The transition from initial injury to chronic pain is mediated by central sensitization, which involves reversible changes to nociceptive neurocircuitry pathways in the central nervous system. Much of our understanding of central sensitization focuses on neurons, whereas the roles of glial cells are poorly defined. Notably, astrocytes that normally maintain and modulate neuronal synapses become reactive and exhibit aberrant Ca²⁺ signals during central
sensitization. However, how these Ca$^{2+}$ signals are generated and how they modulate astrocyte function in central sensitization are unknown. Our objective is to address this by combining powerful genetic tools with a robust model of central pain sensitization in *Drosophila melanogaster*. We assay central sensitization in flies by amputating a single leg and monitoring the response to subnoxious temperatures. Injured, sensitized animals exhibit increased jumping behavior at the subnoxious temperature, whereas animals without injury do not respond to this temperature. Using this assay, we are directly testing the role of metabotropic glutamate signaling in astrocytes, as increased synaptic glutamate is an important feature of nociceptive signaling. Metabotropic glutamate signaling in astrocytes involves Ca$^{2+}$ release from the endoplasmic reticulum (ER) via inositol 1,4,5-triphosphate receptors (IP$_3$R) as well as Ca$^{2+}$ influx via store-operated Ca$^{2+}$ entry (SOCE). Using a Transcriptional Reporter of Intracellular Calcium (TRIC), we found that nerve injury results in enhanced Ca$^{2+}$ signaling in astrocytes. We also found that astrocyte-specific suppression of the SOCE component STIM attenuates nociceptive jumping behavior at one week following nerve injury, suggesting a key role for SOCE-mediated Ca$^{2+}$ signaling in astrocytes during pain sensitization. Our results will bring new understanding to the role of astrocyte signaling in pain sensitization and may suggest novel therapeutic targets for the prevention or treatment of chronic pain.

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**Investigating the C-terminal of KCNT1 to see its regulatory effects on K' and Ca$^{2+}$ homeostasis, inspired from a patient with Brugada syndrome**

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Brugada syndrome (BrS) causes sudden cardiac death (SCD) and abnormal heart rhythms due to several ion channelopathies. To date, over 20 genetic variants have been linked to BrS. KCNT1$^{R1106Q}$ is a mutation found in a BrS patient in 2014, and this is one of the mutations which we are interested in. KCNT1 is a Na$^+$-activated K$^+$ channel producing outwardly rectifying current activated by the binding of cytosolic Na$^+$. We measured the field potential duration (FPD) in KCNT1-overexpressing HL-1 cells. Intriguingly, the KCNT1$^{WT}$ group exhibited a longer FPD, thus we propose that other ions such as Ca$^{2+}$ may also been involved. Though patch recording, we found that KCNT1$^{E411X}$, which lost most of its C-terminus, has a larger current than KCNT1$^{WT}$, implying the KCNT1 C-terminus is pivotal in channel activity regulation. As for KCNT1$^{R1106Q}$, although the current amplitude is not significantly different from KCNT1$^{WT}$, the opening and closing speed is faster. To investigate the roles KCNT1 plays in regulating Ca$^{2+}$ and K$^+$ homeostasis, we made two known *gain-of-function* mutations, KCNT1$^{R428Q}$ and KCNT1$^{A934T}$ as references. Several truncated KCNT1s, namely KCNT1$^{Δ411}$, KCNT1$^{Δ750}$, KCNT1$^{Δ931}$, KCNT1$^{Δ1036}$, KCNT1$^{Δ1146}$, and KCNT1$^{Δ1226}$ were also been made. Using Fura2, we found that the Ca$^{2+}$ influx is increased in KCNT1$^{WT}$, KCNT1$^{R1106Q}$, KCNT1$^{R428Q}$, KCNT1$^{A934T}$, and KCNT1$^{Δ1226}$. The increment is absent in KCNT1s shorter than KCNT1$^{Δ1146}$. In ORAI1 knockdown experiments, we confirm that the store-operated Ca$^{2+}$ entry (SOCE) is
the protagonist of this KCNT1-related Ca\(^{2+}\) influx. To figure out how SOCE is manipulated, we calculated STIM1 puncta in each group, and the density is higher in KCNT1 overexpressing cells. To measure the changes of KCNT1 and its mutated forms in K\(^{+}\) regulation, we utilized PBFI to evaluate the basal cytosolic [K\(^{+}\)]. However, the baseline [K\(^{+}\)] in different groups is obscure. This outcome indicates that KCNT1 may influence SOCE in a K\(^{+}\)-independent way. Our results suggest that the C-terminal tail of KCNT1 may modulate Ca\(^{2+}\) homeostasis in a K\(^{+}\)-independent manner, which gives us an alternative viewpoint on understanding KCNT1-related channelopathies.

P118

**Septins regulate heart contractility through modulation of store-operated Ca \(^{2+}\) entry in cardiomyocytes**

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An important mechanistic underpinning of heart failure is dysregulation of the Ca\(^{2+}\) transport mechanisms that drive cardiomyocyte contractility. Better understanding of cardiomyocyte Ca\(^{2+}\) physiology is therefore essential for development of new heart failure therapies. Recent data from our lab and others demonstrate that store-operated Ca\(^{2+}\) entry (SOCE) is an essential Ca\(^{2+}\) transport mechanism in cardiomyocytes. SOCE refers to extracellular Ca\(^{2+}\) influx that is activated specifically by depletion of sarco/endoplasmic reticulum (S/ER) Ca\(^{2+}\) stores. SOCE is mediated by STIM proteins, which function as S/ER Ca\(^{2+}\) sensors, and Orai Ca\(^{2+}\) influx channels. Importantly, both upregulation and downregulation of SOCE in cardiomyocytes results in defective heart contractility and heart failure, demonstrating that precise SOCE regulation is critical. However, mechanisms that regulate SOCE in cardiomyocytes are poorly understood. To this end, septins have recently emerged as key modulators of SOCE, but functional roles of septins in cardiomyocytes have not been analyzed. Using genetic tools and in vivo analysis of heart contractility in Drosophila, we show that RNAi-mediated suppression of Drosophila septins 1, 2, or 4, previously shown to be required for SOCE activation, results in heart dilation and reduced fractional shortening. These results demonstrate that septins are required for proper heart contractility. Further, these results are nearly identical to the effects of SOCE suppression by STIM or Orai RNAi, suggesting that the effects on contractility may be due to a role for septins in SOCE regulation. Interestingly, septin 7 suppression has been shown to result in SOCE upregulation as opposed to inhibition. Consistent with this, we found that septin 7 RNAi resulted in heart wall thickening and constriction of the heart lumen, similar to cardiac hypertrophy seen with SOCE upregulation due to expression of a constitutively active STIM mutant. The hypertrophy phenotype was partly suppressed by co-suppression of both Septin 7 and Orai, further implicating SOCE dysregulation as the cause of the Septin 7 phenotype. These results collectively suggest that proper heart contractility depends on septin regulation of SOCE in cardiomyocytes.

P119

**Giant recursive cell membrane platforms drive calcium, phosphatidylinositol and kinase signaling**

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High temporal/spatial resolution techniques have provided key insight on nanoscopic, short-lived signaling membrane platforms. However, multiple signaling events, including growth-factor receptor activation, Ca²⁺ signaling, growth/apoptosis induction, require much longer time frames. To reconcile such distinct timescales, we set to explore long-term signaling macromechanics in living cells through super-resolution microscopy, using the transmembrane signal transducers Trop-1/Ep-CAM and Trop-2 as benchmarks. Our findings allowed us to discover that membrane rafts acquire signaling capacity through coalescence of activated growth-inducers and their molecular effectors into macroscopic areas designated as “docks”. Docks spanned several µm and showed lifetimes of hundreds of seconds, shifting current nanodomain-centered signaling models to orders of magnitude-higher dimensional scales. This signaling mode appeared shared across distinct classes of membrane sites, from essentially immobile cliffs to highly-motile ruffles and lamellipodia.

Modulation of focal adhesion dynamics by lipid raft-restricted cAMP signaling within RGCs

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The connectivity of retinal ganglion cell (RGCs) axons into the brain is highly stereotyped and the precise connections are crucial for the function of the visual system. These projections target multiple brain nuclei including two major ones, the superior colliculus (SC) and the dorso-lateral geniculate nucleus (dLGN). The development of these connections involves axon pathfinding and terminal arbor refinement, two processes relying on axon guidance cues. Ephrin-As, a family of axon repellent cues, are critical for the refinement of RGC axons in the SC and the dLGN. We previously demonstrated that EphrinA5-induced repulsion of developing retinal axons requires cAMP signaling restricted to lipid raft. Lipid raft are plasma membrane microdomains enriched in cholesterol and sphingolipids segregating a subset of signaling pathways from the rest of the membrane. However, the downstream effectors of lipid raft-restricted cAMP signals involved in axon pathfinding remain unknown. Efficient axon pathfinding requires an adequate remodeling of cellular adhesion in growth cones. The dynamics of focal adhesions, a major cell adhesion structure, contributes to axon navigation. We hypothesize that the cAMP-dependent variations observed in lipid rafts modulates focal adhesion remodeling. To test this hypothesis, we aim at characterizing cAMP signals in the vicinity of focal adhesions by using subcellular-restricted FRET sensors and genetically-encoded chelators targeting cAMP. We identified focal adhesion-restricted cAMP signals contributing to ephrinA5-induced axonal repulsion. This signal modulates the phosphorylation of the Focal Adhesion Kinase (FAK), one the core component of focal adhesion complexes.

Signaling Pathways in Development and Disease

Decidual Cell Type Differentiation is Homologous to Wound Healing Related Fibroblast Activation

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Decidual cell is a unique placental mammal uterine cell type that forms in support of pregnancy as the maternal interface to the fetus. The differentiation (decidualization) of decidual cell type from
endometrium stromal fibroblasts is a key developmental, as well as evolutionary step to establish pregnancy in placental mammals. It has been shown that decidualization pathways and stress response are evolutionarily related. However, the cellular stress response can be triggered by different external and internal signals. Which signal is the evolutionary origin of this similarity? Using in vitro decidualization model, we provide evidence that decidualization involves activation of the characteristic wound healing related fibroblast activation markers. In addition, we report that TGFb1, a master regulator of fibroblast activation, is required for decidualization. These results support the conclusion that wounding related fibroblast activation is the evolutionary origin of the decidualization process.

P122

**SWI/SNF chromatin remodeling regulates cell cycle exit and promotes cellular invasion in the C. elegans Anchor Cell**

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The success of many metazoan developmental programs relies on the ability of specialized cells to transgress basement membranes (BM). Cancer metastasis also relies on cellular invasion. Chromatin remodeling complexes, such as the SWI/SNF complex, coordinate animal development through broad regulation of gene transcription, ensuring normal cell cycle control and cellular differentiation in lineage-specific and temporally restricted manner. Mutations in subunits of chromatin remodeling complexes are associated with a variety of diseases, including cancer. Here we utilize the in vivo model of *Caenorhabditis elegans* anchor cell invasion (AC) to identify the suite of chromatin remodeling genes that are involved in the promotion of cellular invasiveness. From a large-scale microscopy-based RNAi screen targeting ~400 chromatin modifiers, we identify multiple members of the conserved SWI/SNF complex as new regulators of AC invasion. Through the use of a ratiometric cyclin-dependent kinase (CDK) cell cycle sensor and fluorescent reporters for pro-invasive genes, we demonstrate that the SWI/SNF ATP-dependent chromatin remodeling complex is a critical regulator of AC invasion, with pleiotropic effects at both the level G0/G1 cell cycle arrest and activation of the invasive machinery. Using genome editing and RNA interference, we also show that SWI/SNF contributes to AC invasion in a dose-dependent fashion, with lower levels of activity in the AC associating with aberrant cell cycle entry and greater loss of invasion. Thus, we demonstrate that SWI/SNF is required for AC invasion in vivo both to arrest cell cycle progression and activate pro-invasive genes, which may provide valuable single-cell mechanistic insight into how and why mutations in subunits of the complex are associated with many cancers.

P123

**Nr2f1a maintains nkx2.5 expression to repress sinoatrial node identity within venous atrial cardiomyocytes**

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Nr2f (Coup-tf) transcription factors are conserved regulators of atrial development, with NR2F2 mutations in humans being associated with congenital heart defects (CHDs), including atrial septal defects. Yet, the mechanisms by which Nr2fs function within atrial cardiomyocytes (ACs) are still not understood. To investigate the consequences of Nr2f loss within the atrium, we performed transcriptomic analysis on isolated ACs from wild-type (WT) and nr2f1a mutant zebrafish at 48 hours post-fertilization (hpf), since zebrafish Nr2f1a is the functional equivalent of mammalian Nr2f2 with respect to heart development. Surprisingly, our results showed altered expression of core genes, including increased tbx3a and decreased nkh2.5, that respectively promote and repress sinoatrial node (SAN) differentiation. Quantification of SAN cells using an fgf13a enhancer-trap line, which labels SAN cells, and the myl7:DsRED-NLS transgene, which labels all cardiomyocyte nuclei, revealed a progressive expansion of SAN identity from the venous pole throughout the atrium of nr2f1a mutant hearts from 48 to 96 hpf. Furthermore, high-speed time lapse imaging showed that at 72 and 96 hpf, nr2f1a mutants have slower calcium conduction across the atrium and reduced heart rates compared to WT embryos, consistent with an acquisition of pacemaker identity within nr2f1a mutant ACs. Genetic epistasis using a heat-shock inducible nkx2.5 transgene to restore Nkx2.5 showed that overexpressing Nkx2.5 is sufficient to repress the SAN expansion in nr2f1a mutant hearts. Furthermore, ATAC-seq profiling of open chromatin in isolated ACs identified a putative nkx2.5 enhancer harboring an Nr2f binding site, which we found to be expressed in the atrial myocardium adjacent to the SAN in transgenic embryos. Expression of the putative enhancer is lost in nr2f1a mutants, suggesting that Nr2f1a may directly maintain atrial nkx2.5 expression. Altogether, our results reveal a novel requirement for Nr2f transcription factors in maintaining AC identity at the expense of SAN identity through directly maintaining nkx2.5 expression in vertebrate hearts, which may provide insight into the etiology of arrhythmias and CHDs associated with NR2F2 mutations found in humans.

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Impact of Y64D Mutation on Cellular and Developmental Functions of Rac1

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Rac1 is a highly conserved small GTPase and best known for its role in the regulation of the actin cytoskeleton. This makes Rac1 plays a key regulatory function of nervous system development during embryogenesis that involves multiple cellular events such as neuronal stem cell proliferation and differentiation, axonal growth and stability, formation of synapses and neural tube closure. A missense mutation in RAC1 gene that results in amino acid change from tyrosine (Y) to aspartic acid (D) at position 64 was identified in an individual with developmental delay and neurological disorders. It is currently unclear how this mutation might cause the phenotype observed in the individual. Hence this study aims to explore Y64D mutation effects on the cellular functions of Rac1 and its roles in development. We initially carried out in vitro experiments to investigate how Y64D mutation affects Rac1 function during fibroblast spreading. We found that Y64D enhanced lamellipodia formation, thus phenocopied a constitutively active mutation. To obtain a clearer understanding of how Y64D mutation leads to developmental defects in human, it is therefore desirable to investigate their effects in a model organism. We specifically expressing the Y64D mutation in neurons of fruit fly Drosophila melanogaster to investigate the effects of the mutation in Drosophila development and neuronal growth. Expression
of Y64D resulted in delayed pupation and a severely impaired larval motility. More detailed analysis revealed that this mutation causes changes in the morphology of neuromuscular junctions, disorganisation of the embryonic central nervous system and altered axonal morphology. Expression of Y64D also induced ectopic filopodia during dorsal closure when expressed in the epidermis, suggesting that mislocalisation of Rac1 activity may underlie the defects induced by the mutation. Collectively, our findings provide insights into the cellular and developmental effects of Y64D mutation in RAC1 that are associated with human developmental disorders. These findings bring us closer to understanding the mechanisms underlying these conditions towards developing therapies or diagnostics.

P125

p38 MAPK drives rapid embryonic wound repair by promoting cell growth and survival
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Embryonic wounds are repaired rapidly in a process driven by collective cell movements. Upon wounding, actin and the molecular motor non-muscle myosin II become polarized towards the wound edge in the cells adjacent to the wound. Actomyosin polarization results in the assembly of a contractile cable that coordinates cell movements around the wound. Production of reactive oxygen species (ROS) upon wounding is necessary for cell polarization during wound repair. However, the signals downstream of ROS that drive wound closure are not understood. The serine/threonine kinase p38 MAPK kinase can be activated by oxidative stress to promote cell proliferation during tissue regeneration. However, the embryonic wound response happens in the absence of cell division, and thus, a potential role for p38 in embryonic wound closure is unclear. We investigated the role of p38 during wound repair in Drosophila embryos using quantitative microscopy. We found that p38 was activated in the cells adjacent to the wound. Genetic and pharmacological disruption of p38 resulted in the overexpansion of wounds and a reduced speed of cell migration, with the subsequent delay in wound repair. Strikingly, cytoskeletal polarization and force generation at the wound edge were not affected by p38 inhibition. Instead, when p38 was inhibited a significant number of cells that were not targeted by the wounding procedure were included inside the lesion, resulting in larger wounds. Activation of the p38 pathway accelerated wound repair without affecting the maximum size of the wounds, indicating that p38 also controls the rate of wound closure. p38 is known to regulate cell sizes. We explored the roles of cell growth in promoting rapid embryonic wound repair and found that cell volumes increase as wound closure progresses. Together, our data indicate that p38 may protect cells around the wound from wound-induced damage, and that cell growth may play unexpected roles in embryonic wound healing.

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A human induced pluripotent stem cells based model of schizophrenia reveals defects in vascular endothelial growth factor signaling in the neurovascular niche
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Both the formation of blood vessels in the brain and neurogenesis occur simultaneously during embryonic development, resulting in the formation of the neurovascular niche that brings together physical and molecular signals from both systems. Besides being important for neural and vascular
development, these signals are critical for the formation and maintenance of the blood-brain barrier (BBB). Schizophrenia (SZP) is a neurodevelopmental disease, which is characterized by psychotic symptoms, deficiencies in brain connectivity, and loss of gray matter. Increasing evidence has linked vascular abnormalities to this disease: VEGFA deficiency in postmortem brains and adult blood plasma, hypoperfusion in the neocortex, neuroinflammation, and a BBB dysfunction. The use of human induced pluripotent stem cells (hiPSC) has been expanded as a new strategy to study psychiatric disorders, because they conserve the genetic diversity of donors and recapitulate developmental processes in vitro. Here we characterized the angiogenic profile of different components of the neurovascular niche and evaluated the impact of VEGFA deficiencies in a SZP hiPSC-derived study. Control and SZP derived-hiPSC were differentiated into neural stem cells (NSC), astrocytes and neurons, and brain endothelial cells (BEC). Secretome of neural derived lineages was tested for its angiogenic capacities both in vitro and in vivo. Expression of angiogenic and BBB related proteins was analyzed in BEC, as well as their responsiveness to angiogenic stimuli. Both SZP neural and BEC components showed an imbalance in secretion and expression of several neuro-angiogenic related molecules, particularly those associated with VEGFA signaling. Angiogenic signaling was down regulated in developing neural cell lineages and BEC. BEC revealed an imbalance in BBB related protein expression and were less responsive to VEGFA stimulation. Our findings are consistent with theories of SZP that trace its origins to the brain's development, and to problems that emerge in neurovascular communication that possibly contribute to neural defects and BBB phenotype described in SZP. In this context VEGFA signaling arises as a possible therapeutic approach.

P127

**Hypertonic Dextrose stimulates chondrocyte proliferation and collagen deposition in vitro**

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Osteoarthritis is a disease afflicting more than 30 million Americans with no cure. The disease involves unnatural differentiation of chondrocytes, leading to degradation of cartilage and associated pain. Current treatments such as corticosteroids aim to reduce pain but do not address the cellular onset of the disease. By targeting the inflammatory component of the disease, future therapies slow down and perhaps even reverse osteoarthritis. One promising therapy is hypertonic dextrose injections (HD), a treatment hypothesized to induce a localized inflammatory response leading to increased proliferation and extracellular matrix deposition. Although clinical research suggests HD injections may help in the regeneration of cartilage, the underlying mechanisms are not well understood. We employed Fluorescence Lifetime Imaging Microscopy (FLIM) to capture proliferation and morphology data and Second Harmonic Generation Microscopy (SHG) to quantify collagen deposition using ATDC5 mouse chondrogenic cells. Our utilization of FLIM allowed for tracking via NADH visualization, eliminating the issue of photobleaching with conventional fluorescence microscopy. SHG allowed for continuous quantification of collagen deposition over time without disturbing cellular processes through fixation and staining. We tested the hypothesis that HD is associated with cartilage regeneration via increased proliferation and collagen deposition following treatment administration, possibly through hypertonic shock. Our results indicate HD-treated ATDC5 cells proliferated more than did mannitol- and PBS-treated cells within the study period (p<0.05). HD-treated cells exhibited more collagen deposition than
mannitol- and PBS-treated cells (p<0.05). This study advances the theoretical and empirical understanding of the mechanisms of HD as a treatment for osteoarthritis. Study results support our hypothesis that HD stimulates chondrocytes to proliferate and deposit collagen. Results align with clinical research indicating HD regenerates cartilage. These pre-clinical results, in conjunction with results from multiple clinical trials, provide motivation for further investigation of HD as a promising solution for osteoarthritis.

P128

**From spikes to intercellular waves: tuning intercellular Ca\(^{2+}\) signaling dynamics modulates organ size control**

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Calcium (Ca\(^{2+}\)) signaling is a fundamental mechanism for the propagation of information within cells and is necessary for proper cell function. However, the biophysical mechanisms that govern Ca\(^{2+}\) signaling dynamics between cells remain elusive. *Drosophila* wing imaginal discs are a premier model for studying conserved cell signaling mechanisms within the full organism context. Recent experimental studies in developing wing discs demonstrate the emergence of four distinct patterns of Ca\(^{2+}\) activity occurring on a tissue level. These include single cell Ca\(^{2+}\) spikes, intercellular Ca\(^{2+}\) transients, propagating tissue-level Ca\(^{2+}\) waves, and a global “fluttering” state. Here, we used a combination of computational modeling and experimental approaches to show that there are two different populations of cells in the wing disc pouch connected through gap junctional proteins. We term these two populations “initiator cells” and “standby cells.” Initiator cells initiate calcium signaling within the developing tissue and are outnumbered by standby cells. Initiator cells are predicted to exhibit higher levels of Phospholipase C (PLC) activity and produce more inositol trisphosphate, a key molecule that triggers the release of Ca\(^{2+}\) from the endoplasmic reticulum into the cytosol under agonist stimulation. We show that the strength of hormonal stimulation and the fraction of initiator cells jointly determine the predominant class of Ca\(^{2+}\) signaling activity in a tissue. Further, we demonstrate that single-cell Ca\(^{2+}\) spikes are stimulated by insulin, while intercellular Ca\(^{2+}\) waves are caused by stimulation of PLC as a result of G\(\alpha\)q activity. Our model suggests that the fraction of initiator cells in a developing tissue scales in a power-law fashion relative to tissue size with the fraction of initiator cells in the population decreasing as tissue size increases. Phenotypic analysis of perturbations to G\(\alpha\)q and insulin signaling supports an integrative model of Ca\(^{3+}\) as an dynamic reporter of tissue growth, suggesting that Ca\(^{2+}\) signaling dynamics help to tune the final size of organs. In sum, this study identifies important crosstalk between biochemical growth signals and heterogeneous cell signaling states during the growth of an organ to reach a final size.
Spindle Assembly 1

P129

**Optogenetic control of PRC1 reveals that bridging fibers promote chromosome alignment by overlap length-dependent forces**

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During metaphase, chromosome position at the spindle equator is regulated by the forces exerted by kinetochore microtubules and polar ejection forces. However, the role of forces arising from mechanical coupling of sister kinetochore fibers with bridging fibers in chromosome alignment is unknown. Here we develop an optogenetic approach for acute removal of PRC1 to disassemble bridging fibers and show that they promote chromosome alignment. Tracking of the plus-end protein EB3 revealed longer antiparallel overlaps of bridging microtubules upon PRC1 removal, which was accompanied by misaligned and lagging kinetochores. Kif4A/kinesin-4 and Kif18A/kinesin-8 were found within the bridging fiber and lost upon PRC1 removal, suggesting that these proteins regulate the overlap length of bridging microtubules. We propose that PRC1-mediated crosslinking of bridging microtubules and recruitment of kinesins to the bridging fiber promotes chromosome alignment by overlap length-dependent forces transmitted to the associated kinetochore fibers.

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**Opposing motors provide mechanical and functional robustness in the mammalian spindle**

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The metaphase spindle reaches a steady-state shape, despite continuous microtubule turnover and motor activity. How motors together give rise to the mammalian spindle’s emergent architecture, mechanics, and function remains poorly understood. The motors dynein and Eg5 are each key to bipolar spindle formation: dynein mediates contractile microtubule minus-end clustering, and Eg5 drives extensile microtubule sliding. Yet when both are inhibited, the spindle can still establish its normal shape. What, then, are the roles of these opposing motors? To answer this question, we generate doubly inhibited spindles by knocking out dynein’s microtubule end-targeting factor NuMA and acutely inhibiting Eg5. These spindles not only attain a shape and size indistinguishable from controls, but also enter anaphase. However, under mechanical compression these spindles are more fragile: they deform more easily and fail structurally. Further, they exhibit reduced microtubule dynamics and internal organization, and lagging chromosomes in anaphase. Thus, while these opposing motor activities are not required for the spindle’s shape, they are essential to its mechanical and functional robustness, which we propose occurs through increased spindle dynamics and organization. Together, this work may provide insight into the robustness of other active, self-organizing cellular structures.
Kinetochore movement during chromosome congression depends on the formation of interpolar bundles
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During mitosis, the cell forms a spindle that equally segregates chromosomes into two daughter cells. Precise spatial organization of microtubule bundles and kinetochores is necessary for spindle function, yet it is unknown how unevenly distributed microtubules present during prometaphase organize into precise kinetochore and bridging fibers, important for positioning the kinetochores into the metaphase plate. By using live cell imaging of RPE1 cells stably expressing EYFP-tubulin, we showed that in early prometaphase microtubules are organized in a few unevenly distributed and wide bundles. In contrast, late prometaphase and metaphase spindles are more ordered, consisting of numerous bundles that are similar in width and evenly spaced. Live cell imaging of HeLa cells stably expressing GFP-tagged microtubule crosslinker PRC1 also revealed that in prometaphase, the spindle has a small number of unequally sized and spatially distributed PRC1-labeled overlap bundles, whose number increases either by forming de novo or exploiting the previously formed bundles. Depletion of PRC1 leads to different kinetochore movement during chromosome congression, which delays the formation of a stable metaphase plate. Thus, these results indicate a link between interpolar bundle formation and chromosome movements during congression in human cells.

Large-scale 3D reconstruction of mouse metaphase II meiotic spindles in oocytes of old and young mice and comparative quantification of microtubules characteristics
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The faithful segregation of chromosomes into daughter cells during meiosis is crucial to produce healthy progeny. Any deviation in the natural process of chromosome segregation during cell division can lead to aneuploidy, one of the major causes of miscarriage and infertility. Accurate chromosome separation during meiosis is thought to be driven by microtubules that are connected to special chromosomal regions known as kinetochores. Indeed, defects in kinetochore formation or function compromise chromosome separation and lead to chromosome mis-segregation. Studies on the underlying molecular mechanisms of chromosome segregation indicated that this is an error-prone process. In particular, with increasing maternal age the frequency of mis-segregation increases significantly. The reasons for this age-related increase are however unclear and several hypotheses have been proposed, i.e. cohesion fatigue or kinetochore fragmentation. Hindered by the limits of light microscopy we can neither resolve individual microtubules connecting to the chromosomes, nor their interaction with other components of the spindle. 3D Electron tomography provides the ‘ultimate’ resolution to the lack of structural information. We study the structure of individual chromosomes and their interactions with microtubules by 3D tomography, providing single microtubule resolution, and state-of-the-art light microscopy. Subsequently, computational analysis and simulations are used to develop a mechanical model of how shape and structure of individual chromosomes affect their faithful segregation. Our methodology allows us to detect differences between individual chromosomes and their interactions with
microtubules on a microscale level. We will present our preliminary results on the reconstruction of mouse meiotic spindles in Metaphase II of old and young mice.

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The Impact of Structural Differences Between Chromosome and Microtubule Interactions on Chromosome Segregation
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Accurate segregation of the duplicated genome in mitosis is essential for maintaining genetic stability. Chromosome separation during cell division is thought to be driven by microtubules, connected to special chromosomal regions known as kinetochores. Indeed, defects in kinetochore formation or function compromise chromosome separation and lead to aneuploid daughter cells containing too many or too few chromosomes. Interestingly, recent research has shown that chromosome missegregation is biased. The reasons for this bias, however, has remained unclear. While it is our common understanding that microtubule misattachments drive chromosome missegregation, the detailed interactions of microtubules and individual chromosomes and how this ultimately give rise to missegregation are not well understood. Does a single misattached microtubule already cause missegregation, or do all microtubules have to be misattached, or only a subset? More precisely, is there a threshold of “wrong” microtubule connections that ultimately causes chromosome missegregation? And which other, kinetochore independent, factors are relevant for faithful chromosome segregation? At this point in time we do not have satisfying answers to these questions. In addition to failures in the correct kinetochore microtubule attachment to chromosomes, we can imagine additional factors that could possibly impact chromosome segregation, such as their size, kinetochore size, gene density, interphase nuclear territory, and heterochromatin content. The question of whether these or additional characteristics generate bias in mis-segregation rates remains elusive. Our work concentrates on the hypothesis that bias in chromosome segregation is promoted by structural variations between individual chromosomes. To test this hypothesis, we will quantify structural properties and microtubule binding capacity of (mis) segregating chromosomes by electron tomography. In addition, we will label individual chromosomes with a high missegregation frequency using a modified CRISPR-dCas9 technology. This will enable us to determine spatial-temporal dynamics that might contribute to biased chromosome segregation.

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Understanding the mechanisms that give rise to the mammalian spindle’s response to force using theory and microneedle manipulation
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The spindle is the force-generating structure that drives chromosome segregation at cell division. In mammalian spindles, bundles of spindle microtubules called kinetochore-fibers (k-fibers) pull on chromosomes to move them. While we know nearly all components necessary for spindle function, how k-fibers respond to force and maintain themselves under force remains poorly understood. Our recent ability to exert local force on the mammalian spindle with microneedles provides key information to
answering this question. Here, we use a modeling approach based on Euler-Bernoulli beam theory to identify the minimal mechanical features of the spindle necessary to recapitulate how k-fibers deform under external load. First, we find that force and moment generation at spindle poles are needed to recapitulate observed k-fiber shapes, both with and without external load. Then, we find that crosslinking near kinetochores, which has been experimentally observed, is necessary and sufficient to recapitulate observed k-fiber shapes, assuming no moment generation at kinetochores. By probing the limits of our model under large k-fiber deformations, we infer conditions under high external load beyond which the mechanical integrity of the k-fiber appears compromised, suggesting that structural changes occur under such forces. Finally, we assess the possibility of using our modeling formalism to learn about the applied loads based purely on k-fiber shape analysis. The modeling framework we developed not only helps us understand the mechanisms underlying the spindle’s response to force, but will serve as a quantitative framework for probing how the architecture and dynamics of the k-fiber and its surrounding network give rise to mechanics and function.

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Mechanosensitive cues in the nucleus ensure efficient spindle assembly
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Correct separation and positioning of centrosomes is essential for efficient mitotic spindle assembly. During the initial stages of mitosis, multiple factors drive centrosome migration along the nuclear envelope (NE), to allow the establishment of a bipolar spindle scaffold, which facilitates chromosome capture. Once the spindle is assembled, it then orients according to external cues, to ultimately determine division plane orientation. However, we have recently shown that during prophase, centrosomes do not follow external cues. Instead, they reorient to position on the shortest nuclear axis at nuclear envelope breakdown (NEB). This suggests that an intrinsic property of the nucleus could provide the cues for centrosome positioning during prophase. Here, by using a combination of micromanipulation and microfabrication techniques with live-cell microscopy and computational tools, we show that mitotic chromosome condensation provides mechanical cues on the prophase nucleus that enable dynein loading on the NE. Importantly, by regulating the recruitment of NE-associated dynein, these mechanical cues are essential for correct centrosome positioning during prophase. We further demonstrate that mechanical compression of the nucleus is sufficient to restore dynein loading on the NE when chromosome condensation is impaired, in a NudE/NudEL and SUN1/2-dependent manner. Finally, we present evidence on how the mechanical properties of the prophase nucleus are relevant for chromosome segregation fidelity. Overall, our results suggest that, in addition to the known biochemical pathways, nuclear mechanics play an important role in regulating spindle assembly and mitotic fidelity.
Mammalian Kinetochore-Fibers Regulate Their Length and Dynamics Individually, Independent of Spindle Poles

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At each cell division, the spindle builds itself from tubulin building blocks, with nanometer-scale components giving rise to a micron-scale structure. While many proteins modulating mammalian spindle length are known, how they work together to set spindle length remains poorly understood. To probe the role of spindle architecture in setting the length of spindle substructures, or vice versa, we inhibit dynein to generate spindles whose kinetochore-fibers (k-fibers) no longer focus and connect at poles. We find that these k-fibers have an unchanged mean length, albeit a broader distribution, that they maintain their length over time, and that their microtubules “flux” outward normally. Furthermore, these k-fibers grow back after being laser ablated, and do so by suppressing apparent minus-end depolymerization as they grow, all without a pole. This is in contrast to previous models where the pole and pole-associated forces regulate minus-end dynamics. Thus, k-fibers can individually set and maintain their length without a spindle pole, suggesting a model where spindle length is an emergent property of individual k-fibers, rather than of the whole spindle through the pole.

Structure and function of the ECM

Sculpting of tissues by tension-suppressed degradation of constituting collagen fibrils

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Development of tissue morphology generally depends on matrix and reflects a balance of synthesis and degradation of matrix proteins. Homogeneously tensed tissues and collagenous materials have suggested that collagen degradation is suppressed by strain, but tissues often sustain non-uniform strains that could in principle sculpt the mechanics and morphology of tissue. Here we study such processes in beating embryonic chick hearts and in isolated mice tail tendons. Normal beating hearts are subjected to ~5% peak strain in a spatiotemporally coordinated contractile wave, and the hearts maintain their collagen mass until the contractile strain is suppressed by inhibition of myosin-II. Endogenous MMPs then degrade the collagens of contractility-inhibited embryonic chick hearts within ~30-60 minutes based on addition or not of MMP inhibitors and quantitation by calibrated mass spectrometry (MS). In tendon fascicles, collagen fibrils primarily orient along the tension axis of the tissue but heterogeneous strains and gradients were induced using two different deformation modes i.e. heterogeneous (using three-point bending plus adhesion) and uniaxial, with patterned photobleaching of a fluorescent dye and collagen-binding peptide on fascicles used to measure strain. Microstructure of "cell-free" fascicles was simultaneously imaged using Second Harmonic Generation (SHG) signal where deformed fascicles were exposed to collagenases i.e. purified MMP-1 or Bacterial
collagenase while MS was used to quantify live tendon protein levels. Within physiological strain limits (i.e. ~5-8%), the decrease in fascicle degradation rate (relative to strain-free) was nearly independent of collagenase type despite different cleavage mechanisms, and tissue locations sustaining higher strains showed the degradation rate became almost independent of strain magnitudes. Strain suppression of the degradation rate was independent of tendon deformation mode while degradation dependent collagen organization showed a dependence on initial strain magnitude. Sequestration of collagen’s cleavage sites by strain is most likely because degradation rate is dependent on collagen cross-link level while permeation and mobility of fluorescent collagenase and dextran are strain-independent up to ~5-8% strains. Both tissue systems under heterogeneous strains indicate a degradative loss and sculpting of tissue locations that sustain the lowest strains.

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Normal and chronic obstructive pulmonary diseased human lung fibroblasts reared in serum-free medium produce matrix metalloproteinases and collagen type I providing an experimental system to test novel therapeutics


In the United States, an estimated 16 million adults have chronic obstructive pulmonary disease (COPD). COPD is a condition where the elasticity of the lung is compromised and the lung’s ability to recoil is decreased. As a result, a patient is unable to exhale, thus resulting in decreased blood oxygenation and most importantly, difficulty breathing. The disease process is exacerbated by matrix metalloproteinases (MMPs), specifically MMP 8 and MMP 12, which break down collagen, an integral component of a healthy extracellular matrix (ECM) in a normal human lung. In this study we measure collagen metabolism, MMP 8 and MMP 12 production by human lung fibroblasts (HLFB) reared in serum-free media (normal (NHLFB) and COPD diseased (DHLFB)). Commercially available early passage human lung fibroblasts established from lung tissue, obtained during biopsy, were cultured and passaged in our laboratory through passage five (P5). Both normal and diseased HLFB cells were viable when cultured at subconfluence for 24 hours in serum free medium. We measured production of MMP 8 and MMP 12 in both the secreted (conditioned media ) and incorporated (extracellular matrix) fractions. There were detectable levels of MMP 8 and MMP 12 in both fractions produced by NHLFB and DHLFB through P5. The amount of intact collagen decreased between passage 4 (P4) and P5 in both cell types indicating there is a limit to the passages that can be utilized to measure ECM alterations. NHLFB incorporated 40% more intact collagen into the extracellular matrix than DHLFB and released 37% less into the conditioned media at P4. These relationships persisted in P5 though the percentages decreased to 5% more incorporated and 10% less secreted by the NHLFB. Degraded collagen type I was detectable in both fractions at less than 2% of the total collagen type I produced in both the NHLFB and DHLFB cultures. At both passages, NHLFB made an average of 1% degraded collagen when compared to 0.25% produced by DHLFB. In this study we have demonstrated that normal and diseased HLFB can be cultured in serum free conditions while maintaining their metabolic profile. The production of MMPs and collagens provides an experimental system in which we can test potential therapeutics that may alter the fibrotic response during COPD.
Clusterin modulates lipid biogenesis in trabecular meshwork

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Objective: To study the significance of the secretory chaperone protein, clusterin, in regulating the lipid content and tissue biomechanics of the trabecular meshwork (TM). Biomechanics of TM is important to regulate aqueous humor (AH) drainage and intraocular pressure (IOP), which is closely related to membrane remodeling. Methods: Quantitative-polymerase chain reaction (qPCR), immunofluorescence assays, western blot and lipidomics were performed on human TM (HTM) cells and porcine TM (PTM) cells in culture to assess - a) expression of transcript variants of sterol regulatory-element binding protein (SREBP) and SREBP cleavage- activating protein (SCAP), b) effects of activation of SREBP via clozapine, inhibition of SREBP-SCAP complex via fatostatin, and constitutive clusterin expression using adenovirus clusterin (AdCLU) on - SCAP, proform SREBP (pro-SREBP) in the cytoplasm, active form SREBP in the nucleus (nSREBP), and the regulation of extracellular matrix (ECM) like fibronectin (FN) and collagen (Col1A) in TM, and c) using lipidomics analysis to determine changes in phospholipid and cholesteryl esters under AdCLU. Students t-test was used for statistical analyses and results were significant if p<0.05 with a sample size of n=3-6. Results: Transcript variants of SREBP-F1V1, V2, V3 and - F2V1 and SCAP were expressed in HTM and PTM cells. Immunofluorescence analyses showed that SREBP and SCAP puncta were colocalized on endoplasmic reticulum (ER) membrane and Golgi as visualized by ER marker - KDEL and Golgi marker -GM130. Compared to the control, activation of SREBP by clozapine significantly induced nSREBP1 and increased the FN and Col1A fibril formation. Constitutive clusterin expression lowered the gene expression of SREBP1V2 significantly (p=0.008, n=6). Clusterin significantly induced pro-SREBP1 protein accumulation whereas a significant decrease in nSREBP1 and an appreciable decrease in Col1A fibril formation, which mimicked the effects of fatostatin. Lipidomics analyses revealed constitutive clusterin expression decreased various forms of lysophosphatidyl choline and cholesteryl esters. Conclusion: Our studies identify clusterin as an endogenous lipid modulator in the TM by regulating the SREBP translocation from the ER to nucleus. Therefore, we believe that clusterin can potentially regulate homeostasis of lipid biogenesis. Such lipid modulation can alter the tissue biomechanics via regulating the actin cytoskeleton and ECM proteins in TM. Further studies on the involvement of clusterin in lipid metabolism in TM tissue will have a significant impact on potential regulation of the membrane properties and AH outflow resistance.

Conserved extracellular proteins determine mechanoelectrical transduction channel localization and function in C. elegans touch receptor neurons

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Our senses of hearing and touch are made possible by cells that use ion channels to convert mechanical stimuli into electrical signals. Some of these ion channels are activated by membrane stretch in a force-
from lipids mode, others are activated by deformation of protein tethers in a force-from-filament mode (PMID:26566115). In principle, filaments may be intra- or extracellular and the constituent proteins have direct roles in ion channel activation. Other key proteins indirectly affect sensory function by regulating tissue stiffness or by positioning channels within cells. Despite having identified many or the relevant proteins, we know little about their exact roles in sensory transduction. We used the touch receptor neurons (TRNs) in C. elegans nematodes to investigate the relationship between ion channels and proteins in the plasma membrane, the cytoskeleton, and extracellular matrix (ECM), leveraging the well-characterized MEC-4 channel. MEC-4 localizes to TRN neurites in a distinctive punctate distribution in vivo but not in cultured TRNs in vitro suggesting channel localization depends on factors present in vivo. Time-lapse imaging shows that the MEC-4 puncta are largely immobile in vivo. We examined MEC-4 puncta distribution in mutant animals affecting plasma membrane proteins, cytoskeletal components and ECM proteins to investigate what factors regulate the positioning and stability of the MEC-4 puncta. In doing so, we discovered that the ECM protein nidogen regulates MEC-4 puncta distribution and both behavioral and electrical responses to touch. Nidogen associates with laminin networks and we show that both nidogen and laminin co-localize with MEC-4 puncta. Lastly, we sought to understand more about the mechanobiological significance of the channel’s punctate distribution. By modeling mechanical strain distribution along a neurite in silico, we are testing if either differential stiffness or physical attachment to the ECM at puncta generates localized regions of increased strain that could amplify the mechanical stimulus for ion-channel opening. Future experiments will determine whether these ECM proteins physically anchor the MEC-4 channels, accounting for the immobility of the MEC-4 puncta, and whether such a physical tether is responsible for ion-channel opening in response to a mechanical stimulus.

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Characterizing extracellular matrix of human trabecular meshwork cells isolated from high and low flow aqueous humor regions

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Purpose: Aqueous humor outflow across the circumference of the trabecular meshwork (TM), the primary site of resistance to outflow, is segmental with regions of high-flow (HF), intermediate flow (IF), or low-flow (LF). Ex vivo, these regions have been identified to vary in their molecular signature, in biomechanics, and in response to pressure. Whether cells in these regions are intrinsically different and are capable of depositing distinct extracellular matrices (ECM) is not clear. In this study, we characterized the biomechanics and composition of ECM derived from human TM (hTM) cells isolated from HF and LF regions. Methods: Cultured primary hTM cells [n=3 donors] from HF and LF aqueous humor regions were treated with/without 100 nM DEX, a steroid known to induce ocular hypertension for 30 days. An alkali buffer was then used to remove cells to obtain vehicle control ECM (VehMs) and glucocorticoid-induced ECM (GIMs) for each region. The elastic moduli (stiffness) of VehMs and GIMs were measured using an atomic force microscope. The morphology, organization, and relative expression of ECM proteins: fibronectin (FN), collagen IV (COLIV) and laminin (LAM) were assessed using immunocytochemistry and immunofluorescence techniques. Results: HF and LF-derived ECM showed significant differences in elastic modulus (E). In the presence of Veh, the measured E in LF ECM was two-fold higher than that in HF ECM (p < 0.05). Treatment with DEX significantly increased E in both regions.
in comparison to Veh (p < 0.0001). In addition, LF ECM from DEX treated cells was significantly stiffer than HF ECM from DEX treated cells (2-fold difference; p < 0.0001). In ECM deposited by cells from HF and LF regions, fibronectin (FN), collagen IV (COLIV), and laminin (LAM) expressions were upregulated in GIMs in comparison to VehMs. We also observed morphological differences between GIMs and VehMs. For instance, FN which is a key structural ECM protein was cohesively organized in GIMs but not in VehMs. Notably, LF hTM cells showed greater ECM deposition than HF hTM cells and this exaggeration of ECM in the former was accompanied by cohesive organization of these proteins. **Conclusions:** Our data demonstrates that ECM deposited by cells from HF and LF regions are intrinsically different in terms of biomechanics and morphology. Such biophysical differences suggest intrinsic differences in functional properties of these cells that could underlie response to stimuli and dictate pathological states.

**P142**

**Collagen Fibrils Formation from Collagen-encapsulated Nanoliposomes Stimulated by Electric Fields**

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Collagen is one of the prominent scaffolding components of the extracellular matrix that can turn into collagen fibers through self-assembly mechanisms. Recently, we found that the collagen fiber formation process can be stimulated by an electric field in an acidic condition through the aggregation of tropocollagen. Therefore, we designed an experiment to form collagen fibers by encapsulating collagen molecules in nano-sized liposomes (Lip-Col) and confirmed that the fibril could be formed from Lip-Col under an electric field in an acidic microenvironment. First, we measured the average particle size, encapsulation efficiency, and stability of Lip-Cols at various pH conditions. When applying an alternating current (AC) of 10Hz-1V, collagen fibrils were appeared within ca. 20 minutes. Fluorescence imaging results showed that after applying an electric current for 2 hours, the collagen fibrils were formed in a matrix-like structure at pH 3, while they were in an aggregated form at pH 2. Based on these results, these Lip-Cols can be utilized as a drug delivery system for anti-aging or wound healing agents.

**keywords:** collagen fiber, collagen fibril, liposome, electric field

**P143**

**Release of the oocyte from its extracellular matrix shell, the zona pellucida, is mediated by metalloproteinase activity**

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Spontaneous meiotic maturation of the oocyte occurs after ovulation is triggered or upon its removal from the follicle. This also initiates the detachment of its extracellular matrix shell (the zona pellucida, ZP) from the oolemma. Detachment occurs progressively over the first hours of maturation, but it is unknown what initiates it. We hypothesize that the inner layer of ZP proteins remain in a transmembrane form maintaining attachment to the oocyte, and that their cleavage is mediated by a
yet-unknown peptidase. We adopted a bioinformatics strategy to generate candidates for a peptidase responsible for detachment. We compared a public RNAseq dataset (GSE70116) for transcripts in fully grown oocytes with the MEROPS peptidase database to find peptidases expressed in oocytes. Gene Ontology terms “plasma membrane” and “extracellular space” focused our results to appropriate localization. This generated 39 possible candidates across 6 families of peptidases. We then evaluated small molecule inhibitors selective to each class of candidates using an osmotic shock assay to determine ZP-oocyte attachment. We found that the matrix metalloproteinase (MMP) inhibitors batimistat or marimistat (selective for the M10 and M12 families) entirely inhibited detachment of the ZP from the oolemma except for localized detachment where the polar body extruded. Oocytes matured in the presence of inhibitor at rates similar to controls, and parthenogenetic activation rates were also similar. The effect on the transmembrane form of zona pellucida 3 protein (ZP3) was evaluated using immunofluorescence with an antibody that targets an epitope distal to the putative cleavage site. A brief incubation in acid Tyrode’s solution removed any cleaved ZP3 to reveal the remaining transmembrane subset. Significant loss of transmembrane ZP3 protein was noted by 90 minutes following oocyte isolation with further significant decreases in signal occurring at 2.5, and 4 hours post isolation. Inhibition of metalloproteinase activity prevented ZP3 loss. A more selective inhibitor against the M10 family of MMPs (GM6001) did not reproduce the persistent attachment phenotype seen with batimistat or marimistat; however a selective inhibitor of ADAM10 in the M12 family (GI254023X), which was one of the most highly expressed protease candidates in the transcriptomic screen, did produce the same suppression of detachment seen with the broader inhibitors. We conclude that the M12 family of MMPs are responsible for the ZP detachment from the oocyte, and that ADAM10 is a likely candidate for mediating this process.

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**Formation of Controllable Fibronectin Network for 3D Cell Culture**

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Extracellular matrix is a connective fiber network supporting cells living to its surrounding environment. Particularly, in basal region of skin tissue, a multi-component extracellular matrix, mainly composed of fibronectin (FN), laminin (LAM) and collagen (COL) are formed in forms of fibers. That provide the physical and mechanical stability, and intercellular communication necessary for tissue morphogenesis and homeostasis. Here, we harnessed the fibrillogensis to architecture the multi-component extracellular matrix. We simultaneously and subsequentially manipulated the ECM components network by depositing various ECM molecules (FN, LAM and COL) to drive cellular ECM synthesis. We found that cell-derived FN network could be grown from all of the ECM components, pre-deposited on a surface. Although the FN and COL have been known to induce FN fibrillogenesis reciprocally, however, interestingly, we also found that FN fibrillogenesis could induced by LAM. The unfolded and extended FN fibril formation were confirmed by fluorescence resonance energy transfer (FRET) assay. We believe that this study could provide a controllable multi-component ECM network fabrication as a novel platform for cell-mimicking ECMS in tissue engineering.
Therapeutic Targets

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**PLEKHA4 Promotes Wnt/β-catenin Signaling-Mediated G1/S Transition and Proliferation in Melanoma**

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Melanoma patients incur substantial mortality, despite promising recent advances in targeted therapies and immunotherapies. In particular, inhibitors targeting BRAF-mutant melanoma can lead to resistance, and no targeted therapies exist for NRAS-mutant melanoma, motivating the search for additional therapeutic targets and vulnerable pathways. Here, we identify a regulator of Wnt/β-catenin signaling, PLEKHA4, as a factor required for melanoma proliferation and survival. PLEKHA4 knockdown in vitro leads to lower Dishevelled levels, attenuated Wnt/β-catenin signaling, and a block of progression through the G1/S cell cycle transition. In mouse xenograft models, inducible PLEKHA4 knockdown attenuated tumor growth in BRAF- and NRAS-mutant melanomas and synergized with the clinically used inhibitor encorafenib in a BRAF-mutant model. As an E3 ubiquitin ligase regulator with multiple lipid and protein binding partners, PLEKHA4 presents several opportunities for targeting with small molecules. Our work identifies PLEKHA4 as a promising drug target for melanoma and clarifies a controversial role for Wnt/β-catenin signaling in the control of melanoma proliferation.

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**Paving the Way: Bone Morphogenetic Protein 9 (BMP9) and NELL-like protein 1 (NELL-1) causes over-expression of SHH gene in Adenoviral form**

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SHH expresses Shh, which is integral to development processes in humans, specifically in neurogenesis and organogenesis. A deficiency of Shh (low to no SHH expression) contributes to severe neurodevelopmental disorders that significantly abbreviate infants’ lifespan due to the limited cognitive capacity that follows, making it difficult for children to perform vital and involuntary actions. delayed formation of areas of brain. Protein BMP9, especially when accompanied by NELL-1, has been known to play significant roles in the general development of the human body, with BMP9 having a high applicability to multiple types of development, including neurogenesis. The focus of this experiment was to find a way to increase SHH expression in order to compensate for the little SHH expression that characterizes these developmental disorders, and the increased SHH expression can lead to increased production of Shh protein production. The efficiency of BMP9 as the sole agent in increasing SHH expression versus with NELL1 was tested to determine the most viable candidate for significant overexpression and strong up-regulation; NELL-1 was not tested alone due to its little impact on development individually. iMEFs (immortalized mouse embryonic fibroblasts) were the primary subject of the study, the cell type used when quantifying gene expression and in infections. Adenoviral forms of BMP9 and NELL1 were used to infect iMEFs, and a TqPCR was later performed. Following a TqPCR analysis, the cycle threshold values indicated that, for SHH, the most mRNA was present in Ad-
BMP+NELL1. Greater mRNA amounts indicated increased gene expression. The fold change for each pairing of the qPCR was then determined, and it was found that the fold change for Ad-BMP+NELL1 was the highest out of all the SHH fold changes meaning that expression was up-regulated by Ad-BMP+NELL1, meaning SHH was overexpressed by the treatment. This information presents a breakthrough in neurodevelopmental disorder research as future studies can investigate treatment types by exploiting this information to ensure the effects of such disorders are suppressed using the knowledge that the increased SHH expression prompted by the treatment will logically lead to increased Shh protein amounts, which is integral to the treatment of such disorders.

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Development of an RPE specific in vitro Stargardt disease model
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PURPOSE: The retinal pigment epithelium (RPE) is a polarized tissue that supports photoreceptors of the eye, transports nutrients, secretes growth factors and cytokines and is directly associated with healthy vision. Our lab studied the surface proteome of RPE by cell surface capturing technology which revealed the presence of ABCA4 on the surface of RPE. We focused on the role of the ABCA4 protein as mutations in this protein have been associated with various retinal degenerative diseases including the Stargardt disease. To specifically understand the function of ABCA4 protein in RPE, we developed an in-vitro Stargardt disease model using ABCA4−/− induced pluripotent stem cells (iPSC)-derived RPE. METHODS: We used CRISPR-CAS9 technology to generate ABCA4−/− iPSCs which were differentiated into RPE. These RPEs were matured on transwells (semi-permeable membrane) for 6 weeks before analysis. The RPE monolayers were evaluated by structure, morphology and function by measuring the transepithelial resistance, electrophysiology and ability to digest photoreceptor outer segments. ABCA4 knockout was confirmed by RT qPCR, dd PCR, immunostaining and Sanger sequencing of the gDNA. RPE cells were treated with photoreceptor outer segments for a period of 8 days or by human complement for 48 hours, and sub-cellular lipid deposits were evaluated in those cells by immunostaining to outline the sequence of pathogenesis of Stargardt disease. RESULTS: ABCA4−/− iPSC-RPE displayed normal electrophysiological response and structural properties compared to the control derived iPSC-RPE. The knockout ABCA4 RPEs exhibited reduced ability to digest photoreceptor outer segments and exhibited sub-cellular lipid accumulation while exposed to photoreceptor outer segment or human complement regimen. CONCLUSIONS: Our results indicate that ABCA4 plays an important role in RPE function and loss of ABCA4 leads to the functional defects in RPE cells contributing to Stargardt disease pathogenesis.

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Unraveling the Molecular Determinants of Keloid Formation and Variable Effects of Steroid Treatment using Patient Derived Keloid Fibroblasts
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Keloids are fibrotic skin lesions that occur in susceptible individuals due to abnormal wound healing and are characterized by excessive fibroblast proliferation and collagen deposition. Dark-skinned individuals like African-Americans have high incidence of keloids, forming it 15 times more frequently than lighter-
skinned people, suggesting that genetic and/or epigenetic factors contribute strongly to keloid disease. Keloids are commonly treated using steroids, although the response is highly variable. So far, the molecular mechanisms that drive keloid formation and determine their response to therapy are unclear. To address this issue, we performed RNA sequencing of normal and keloid fibroblasts following steroid treatment to uncover mechanisms involved in keloid pathogenesis and their response to steroids. First, we used Principal Component Analysis (PCA) to visualize variation between keloids and normal fibroblasts following steroid treatment. Keloid fibroblasts from multiple patients clustered together, suggesting that keloids share very similar transcriptional profiles, while normal fibroblasts did not cluster together. Interestingly, keloids fibroblasts were also grouped in distinct clusters upon steroid treatment, whereas normal fibroblasts did not show this effect. Specific patterns of differential transcript usage (DTU) have been associated with various human disorders, but have not been studied in keloid disease. Hence, apart from identifying the up- and downregulated genes in keloid fibroblasts, here we have identified 270 differentially expressed (DE) and DTU transcripts using K-means clustering coupled with gene ontology (GO) analysis. We found the clustered transcripts showed shared biological functions. To investigate the biological impact of DTU, we performed isoform function (ISOGO) analysis. This revealed that IGFBP2 gene expression and one of its isoforms is upregulated in keloids, which was confirmed by qRT-PCR. Increase in this isoform is predicted to affect the negative regulation of cell migration and positive regulation of insulin-like growth factor receptor (IGFR) signaling. Conversely, IGF2BP1 gene and one of the isoforms were downregulated in keloids, which may lead to the stabilization of the IGF2 mRNA. The SOD2 gene is also downregulated in keloids, which is consistent with keloids exhibiting higher oxidative stress in our assays. Furthermore, we found that a SOD isoform that is upregulated in keloids may be associated with a loss of its antioxidant activity. In conclusion, the combination of our transcriptomic and cell biological data have revealed several pathways that could have important implications for our understanding of the mechanisms contributing to keloid formation and their sensitivity to steroids.

**Tubulin Isotypes**

P149

**Cold-adapted tubulin mutations reveal interplay between intrinsic activity and MAPs**

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Microtubule networks coordinate the intrinsic activity of tubulin proteins and extrinsic regulators to generate dynamic polymers that drive diverse cellular process. Although the roles of MAPs in regulating microtubule dynamics are well-established, we have a relatively poor understanding of how changes in tubulin’s intrinsic activity amplify or dampen these roles. We hypothesize that the functional impacts of MAPs are determined by changes in tubulin’s intrinsic activity. To test this hypothesis, we used a genetic approach to identify synergies between tubulin activity and MAPs. We first investigated how the lateral interfaces between tubulin subunits contribute to microtubule dynamics. We guided our investigation by first identifying unique amino acid substitutions within the lateral interfaces of β-tubulins of Antarctic species that maintain dynamic microtubules at low temperatures. We generated mutations in the yeast β-tubulin, TUB2, to mimic the H1-S2 and M loops of Antarctic β-tubulins and found two single amino acid substitutions, S278G in the M loop and S56D in the H1-S2 loop, that individually increase microtubule dynamicity. We then used these mutants to determine how altering the intrinsic activity of
tubulin changes microtubule function and requirements for different MAPs. We find that the S56D substitution slows the proliferation of yeast cells, causes mitotic spindle defects at high temperatures, and increases the requirement for the yeast EB protein, Bim1. In contrast, a β-tubulin mutant, tub2-C354S, known to stabilize microtubules exhibits stable mitotic spindles at high temperature and requires a different set of MAPs. Our results identify tubulin’s lateral interface as a key site for regulating dynamicity and suggest a model in which different organisms or cell types can alter microtubule network function by shifting the blend of tubulin isotypes and MAPs.

P150

Tubulin Isotypes Play Specialized Roles for Efficient Spindle Positioning During Yeast Mitosis
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Microtubules (MTs) are, dynamic cytoskeleton filaments polymerized from tubulin, a heterodimer of alpha and beta-subunits. The vast majority of eukaryotic organisms express multiple genes encoding variants or isotypes of α- and β-tubulin. Despite the critical and diverse roles of microtubules across eukaryotic biology, the functional relevance of tubulin isotypes remains largely an open question. Their clinical importance is further revealed by human neurological and fertility disorders caused by mutations in specific isotypes. We combined genetics, cell biology and quantitative fluorescence imaging to address the question of tubulin isotypes in the tractable budding yeast model. Pioneering studies using deletion or overexpression of the two alpha isotypes (Tub1 & Tub3) proposed they are functionally interchangeable. Nevertheless, the contribution of each isotype to cellular MT function is essentially unknown. We implored advanced genetic techniques to engineer isogenic yeast strains expressing single isotypes with tubulin levels comparable to total α-tubulin in wildtype cells. We find that cells lacking either isotype but expressing proper tubulin levels exhibit compromised MT function such as opposite sensitivities to microtubule poisons, altered regulation of microtubule dynamics, and defects in mitotic spindle organization. A major strength of the yeast system is genetic tractability. Using genome-wide genetic analysis, we find that each isotype co-evolved with the two major mechanisms responsible for spindle positioning during mitosis. The yeast spindle is positioned by two distinct pathways, and our results demonstrate that Tub1 and Tub3 differentially recruit protein factors required for proper functioning of the Dyn1- and Kar9-dependent pathways, respectively. These results provide among the most compelling support that tubulin isotypes contribute specialized microtubule functions. Altogether, our study unveils novel mechanistic insights into how isotypes allow conserved MTs to function in diverse cellular processes.

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Functional Properties of the Vertebrate bIII- and bV-Tubulin Isotypes and an Unexpected Similarity to Cephalopod Tubulins.
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Tubulin isotype families appear to be phylum-specific. In vertebrates, the βIII isotype has certain positions in its sequence at which it differs from the βI, βIIA, βIIB, βIVA and βIVB isotypes. These differences are highly conserved in evolution. At some of these positions, βIII and βV are identical. βIII
appears to have at least three functionally unique properties. First, the assembly of αβIII into microtubules is resistant to superoxide anion, unlike the case with αβII and αβIV. This could be because βIII has S239 instead of the easily oxidized C239 and may explain why βIII is most concentrated in tissues with high levels of free radicals, such as neurons and high-grade cancers. Second, βIII reacts with glutathione, whereas βII reacts very little and βI not at all. This may arise from βIII having C124, very close to the almost universally conserved C127 and C129. Third, when mixed with even small amounts of other β isotypes, βIII forms very dynamic microtubules. One could imagine this being important to cancer metastasis and neuronal plasticity and may explain why patients with high-grade breast cancer had mutations in βIVB in which they replaced the wild-type amino acids with those that βIII has at those positions: C124, N126, V155, I189, A218, S239, A275, T315, A332, I333, S335, V351, S365. Of these 13 positions, 8 also occur in βV. Little is known about structure-function correlations in βV. However, βV occurs in higher quantities in the nose and lungs, which also produce NO, and since it is apparently not found in fish, may have appeared when these organs arose. In addition, βIII and βV differ from other β isotypes in having V37, S55 and S56. It is interesting that, although most of these positions, especially C124, that are almost unique to βIII, are virtually never found in other eukaryotes with the exception of the cephalopod molluscs, which at these positions resemble βIII and βV more than they do any other eukaryotic β-tubulin. Given that cephalopods may be the most intelligent invertebrates—one of them, the octopus, can rejuvenate its own brain—these apparent coincidences suggest that a study of cephalopod tubulin could reveal much about structure-function correlations in βIII and its role in neurons and could also be useful in understanding the role that βIII plays in cancer.

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Alanine scanning of dinitroaniline/phosphorothioamidate binding site on α-tubulin of Plasmodium species

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Human malaria is a complex disease caused by such Plasmodium species as P. falciparum, P. vivax, P. ovale and P. malariae. Previous studies have shown that dinitroanilines and phosphorothioamidates compounds, depolymerazing specifically plant microtubules, are also active against P. falciparum and may act as antimalarial drugs. Therefore these compounds are considering as one of most priority groups for the search of new antimalarial agents. Our investigation was oriented on identification of amino acid residues and interactions, predetermining existence of joint site and similar interaction of α-tubulin from P. falciparum and other species with dinitroaniline and phosphorothiamide compounds. Alanine scanning mutagenesis indicated that two key (Arg2, Val250) and one minor (Glu3) residues are involved in the binding of both groups of compounds - dinitroanilines and phosphorothioamides. At the same time it was revealed, that two minor residues (Asp251, Glu254) interact only with some members of dinitroaniline group. Despite existence of the general mechanism of dinitroaniline and phosphorothiamide binding, alternative interactions within the previously defined site are enough realistic. We assume that these differences can contribute to total binding energy and predetermine variations in binding of studied compounds in the site. Our data indicate that in the case of dinitroaniline compounds such differences may be stronger than in the case of phosphorothioamides, what opens additional possibility for the design of new antimalarial drugs.
α-tubulin protein levels are tuned to ensure cells buffer against β-tubulin toxicity
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The microtubule cytoskeleton undergoes major reorganizations during mitosis, process extension and ciliogenesis. To do this, cells must rapidly increase the supply of tubulin heterodimers, and, accordingly, the levels of α- and β-tubulin protein monomers. Therefore, a fundamental question is if cells regulate the levels of α- and β-tubulin and how cells might regulate that balance. Previous work has provided insights into how α- and β-tubulin are individually regulated. However, these studies do not identify a mechanism that mediates regulation between α- and β-tubulin to maintain stoichiometry for heterodimer assembly. We address this question by investigating how α- and β-tubulin balance at the level of gene copy number, RNA splicing, and protein turnover. We find that yeast cells maintain excess α-tubulin, compared to β-tubulin; and that excess α-tubulin limits the accumulation of β-tubulin. Decreasing α-tubulin gene copy number by knocking out one α-tubulin encoding gene results in lower levels of α-tubulin protein, and slightly higher levels of β-tubulin protein. We find that higher levels of β-tubulin is toxic and lead to slower cell doubling times and inability to form colonies. However, α-tubulin protein levels do not respond when β-tubulin is decreased by knocking out one gene copy. Interestingly it appears that α-tubulin has a shorter half-life β-tubulin which could require higher levels of α-tubulin production. One mechanism *Saccharomyces cerevisiae* are able to increase gene expression is through retaining an intron in the gene. Both α-tubulin genes in *S. cerevisiae* include introns. We investigated how the two α-tubulin isotypes in budding yeast coordinate expression to maintain the ratio. We find that the intron is an important regulator of α-tubulin expression and isotype balance. Finally, we provide evidence for toxicity associated with excess β-tubulin. Our results support a model in which the cell only maintains a level of α-tubulin protein that is needed for the appropriate level of β-tubulin and necessary to prevent detrimental effects associated with excess β-tubulin.

Determining the functional significance of beta-tubulin isotypes in microtubule polymer composition and chemoresistance
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Cancer cells display altered expression levels of tubulin isotypes, but it is unclear whether these changes underlie unique dependency on remodeling of the cytoskeletal network for proliferation and chemotherapy response. Tubulin isotypes are α and β proteins, encoded by different genes, that vary in amino acid sequence and cell-type specific expression patterns. Over expression of the β-tubulin-3 (TUBB3) isotype, up 5-10% of the total tubulin pool, is seen in several cancers and predicts resistance to taxane based therapies. Despite knowing the RNA and protein levels of tubulins within cells, it remains to be investigated what the actual levels of specific isotypes are within microtubule polymers, where different isotypes may be incorporated at different rates. A major hinderance to investigating specific isotypes is antibody specificity. Using a GFP over-expression model we validated a panel of isotype-specific antibodies through immunoblotting. This allowed us to measure the differential incorporation of isotypes into polymerized microtubules. Using a ratio-based analysis of immunoblotting we analyzed soluble tubulin vs polymerized tubulin to test whether TUBB3 is incorporated into microtubules at
higher rate than other isotypes. To understand how tubulin isotypes impact microtubule activity in cells, we measured microtubule dynamics in a panel of different cell lines with varying levels of TUBB3 expression, using a combination of in vivo imaging experiments and in vitro reconstitution. This allowed us to examine tubulin activity in the presence or absence of any external regulators. Lastly, to further elucidate the role of TUBB3 in response to the chemotherapeutic drug paclitaxel, we used a panel of cell lines with varying levels of TUBB3 expression to measured response to physiological doses of paclitaxel through the formation of multipolar spindles and changes in mitotic index in a panel of different cell lines with varying levels of TUBB3 expression. Lastly, to understand how tubulin isotypes impact microtubule activity in cells, we measured microtubule dynamics in different cell lines using a combination of in vivo imaging experiments and in vitro reconstitution. This allowed us examine tubulin activity in the presence or absence of any external regulators. These studies will define the impact of TUBB3 in cancer cells and give new insights into how changes in isotype expression translates to polymer composition and overall activity of the microtubule network.

Tumor Invasion and Metastasis: Cell migration

P155

The Role of Potassium Channel-driven Bioelectric Signaling in Breast Cancer Cell Migration

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Metastasis, the dissemination of primary tumor cells to other organs in the body, is the leading cause of death in breast cancer patients. There is a critical need to better understand the mechanisms that drive local cell invasion to better predict and treat metastatic disease. Bioelectricity is an important mediator of many aspects of cell behavior, such as migration during wound healing, but is poorly understood in the context of migration during metastasis. The bioelectric state of a cell is determined by the charge across a cell membrane (resting membrane potential; RMP) which in turn is regulated by expression of ion channels such as potassium (K⁺). Although with other channel types, K⁺ channels regulate the RMP causing the cell to become more negative (hyperpolarized) or less negative (depolarized). However, the link between K⁺ channel activity, RMP, and migration has not been explored. We found that the RMP of highly metastatic triple-negative breast cancer cell lines is more sensitive to changes in extracellular K⁺ concentration compared to other ions, suggesting that K⁺ flux is important in establishing their RMP. Interestingly, K⁺ channel expression is dysregulated in breast cancer patients and associated with increased malignancy. Based on these results, our objective is to understand the role of bioelectric signaling driven by K⁺ channels in breast cancer metastasis. We used two triple-negative breast cancer cell lines, MDA-MB-231 (highly metastatic) and MDA-MB-468 (poorly metastatic) and compared them to the immortalized breast epithelial cell line MCF10A. To manipulate the RMP we engineered stable expression of two different types of K⁺ channels: Kir2.1 (inward-rectifying) and Kv1.5 (voltage-gated) in these three cell lines. We found that expression of either K⁺ channel caused RMP hyperpolarization that was accompanied by increased 2D and 3D migration in the MDA-MB-231 highly metastatic line, but no change in the less metastatic MDA-MB-468 or normal MCF10A line. This suggests that breast cancer cell migration is driven by the activity of K⁺ channels. We are currently performing RNA sequencing to better understand how changes in K⁺ flux impact gene expression to regulate cell migration. This work will
increase our understanding of the mechanism of bioelectric control of cell migration and metastasis leading to improved means of predict and treat metastatic disease.

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**Metabolic requirements for cell motility in confined 3D environments**

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Cell motility requires ATP-intensive processes such as rapid cycling of ATP-binding actin monomers during cytoskeletal rearrangements and ATP hydrolysis to fuel actomyosin contractility. Furthermore, cells migrating *in vivo* encounter openings as small as 2 μm in diameter, meaning that deformation of the large and rigid cell nucleus becomes a rate-limiting step and could impose an additional metabolic burden. Studying single cell energetics in real-time can aid in understanding how cells dynamically employ metabolic strategies during migration and how this can be affected by physical obstacles, nutrient availability, or therapeutic intervention. We developed an improved fluorescent biosensor (PercevalHR2) for ratiometric imaging of ATP:ADP in living cells. PercevalHR2 can be imaged with a single, cell-tolerable excitation wavelength and works over a wide range of physiological ATP:ADP ratios. PercevalHR2 was expressed in cancer cell lines and imaged during the migration of cells through small openings in a 3D microfluidic device. We determined that the ATP:ADP ratio is heterogeneous within cancer cell lines; intriguingly, the subpopulation of cancer cells with a higher ATP:ADP ratio exhibits faster migration through confined spaces. To further investigate the metabolic pathways supporting confined migration, we applied a panel of inhibitors impacting ATP production and metabolic signaling. This revealed that the mitochondrial-targeting antidiabetic drug Metformin specifically impairs confined migration without decreasing cell speed through wider channels. This was accompanied by decreased force generation and adhesion size without significant change in ATP levels. Surprisingly, inhibition of glycolytic ATP production did not impede migration through the microfluidics devices despite severely diminishing proliferation, supporting that the metabolic phenotypes that fuel highly invasive cell behaviors are likely to be distinct from the pathways characterized to promote tumor growth. To further confirm the importance of mitochondrial metabolism in confined cell migration, we generated cell lines with loss of mitochondrial function due to destruction of mitochondrial DNA, resulting in altered cell morphology and impaired migration abilities. Taken together, our results indicate that the physical environment of the cell influences metabolic requirements for motility, and mitochondrial energy production supports confined migration. Metabolic heterogeneity could give rise to cancer cell subpopulations with enhanced confined migration abilities and successful cancer therapy may require distinct strategies to target both the metabolic pathways supporting tumor growth and the pathways that enhance invasion and metastasis.

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**Impact of Tumor Cell Plasticity and Alterations of the Immune Cell Landscape on the Metastatic Capability of Breast Cancers**

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Metastasis, the growth of secondary tumors in distant organs, is the major cause of cancer-related deaths due to insufficient therapeutic effects of conventional treatment. Individual tumor cells exhibit heterogeneous features and growth potentials within the same tumor that may influence metastasis formation. The underlying mechanisms of why some tumor cells can give rise to metastases, whereas others cannot, are remarkably poorly understood. During the metastatic cascade, tumor cells adapt their phenotype to various microenvironments that are distinct from their original site and are “shaped” by different immune and stromal cell populations. Metastatic tumor cells are heterogeneous and different from the primary tumor cells and therefore, we hypothesize that a successful metastasis formation is dependent on the microenvironment at the distal tissue. Our study aims to better understand the tumor-immune cell axis: how tumor heterogeneity influences immune cell reprogramming and how, in turn, altered immune cells impact metastasis formation. We established patient-derived xenograft models of breast cancer with different metastatic potential and preserved tumor heterogeneity. Using a novel multiplexing approach for high-throughput single-cell RNA sequencing (MULTI-seq) we capture the heterogeneity of primary tumor and metastatic cells and their associated immune cells. We characterize intra-tumor heterogeneity and show that the plasticity of tumor cells is beneficial for the formation of metastasis. We identify markers enriched in metastatic cells and marker gens for different states of epithelial-mesenchymal transition. Our data show that while primary tumors from different patients share a similar myeloid phenotype, metastasis-associated myeloid cells show distinct expression signatures related to the metastatic capability of the primary tumor. Additionally, we find subsets of myeloid cells to be enriched in metastasis of highly aggressive tumors which may promote tumor plasticity-driven metastasis. Thus, these innate immune cells provide a potential target for immune cell therapy that may inhibit tumor cell plasticity and thereby the development of metastasis.

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S100A4 regulates matrix degradation in breast cancer cells
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The ability of tumor cells to invade neighboring and distant tissues depends on their ability to form actin-rich invasive structures called invadopodia. These structures possess proteolytic properties, which allows them to degrade the extracellular matrix (ECM). S100A4, a member of the S100 family of calcium-binding proteins, binds target proteins such as non-muscle myosin II in a calcium-dependent manner. S100A4 is expressed in a wide variety of solid tumors, and its expression correlates with an increased incidence of metastasis and a poor prognosis. Despite its well-established role in promoting a metastatic phenotype, the role of S100A4 in invadopodia formation and matrix degradation has not been explored. We now show that loss of S100A4 blocks matrix degradation in MDA-MB-231 triple negative breast cancer cells and Src-transformed murine fibroblasts, both well-established models of invadopodia-mediated matrix degradation. In MDA-MB-231 cells, S100A4 knockdown cells decreases basal and agonist-stimulated phosphorylation of Src and the invadopodial proteins cortactin and paxillin, inhibits invadopodia maturation, and decreases MMP9 secretion. Moreover, loss of S100A4 blocks STIM1-induced matrix degradation in MDA-MB-231 cells, suggesting that S100A4 regulates matrix degradation.
downstream of STIM1/Orai1. Our data suggest a novel role for S100A4 in triple negative breast cancer cells in the regulation of matrix degradation in response to calcium entry.

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**Proliferation and migration of MCF-7 breast cancer cell line in the presence of bisphenols**

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Bisphenols are common plasticizers that share a similar structure to estrogen. Bisphenols can bind to estrogen receptors and disrupt both classical and non-classical pathways to elicit a cellular response. The endogenous hormone, estrogen, is known to modify enzyme function and elicit cell proliferation of certain cell types, including breast cancer cells. Similar effects, including an increase in metastasis, are seen in breast cancer cells exposed to bisphenol A (BPA). Although many industrial companies have removed BPA from the manufacturing process, they are often replaced with other structurally analogous bisphenols. We hypothesize that these bisphenols increase metastasis and proliferation of cells due to structural similarities, enabling binding to estrogen receptors. Through the use of MTT assays and wound healing assays, we have shown that BPA structural analogs may also modify behavior of MCF-7 line of human breast cancer cells.

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**Characterizing the role of podocalyxin in collective breast tumor invasion**

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High expression of podocalyxin, a CD34-like single-pass transmembrane sialomucin, has been shown to correlate with poor disease outcome in a number of solid tumor types, including colorectal, ovarian, pancreatic and breast cancers. We had previously identified that high podocalyxin expression in invasive ductal breast carcinoma was an independent indicator of poor survival (Somasiri et al., 2004, Cancer Res. 64:15). Importantly, further analysis of these tumor samples revealed that this decrease in survival occurred without the loss of membranous, junctional E-cadherin, suggesting that these tumors may have invaded collectively without necessitating an overt epithelial to mesenchymal transition (EMT). Experimentally, forced over-expression of podocalyxin in polarity-disrupted human MCF7 breast cancer cells drives the formation of collectively invasive orthotopic xenograft tumors and elongated, cohesive, and E-Cadherin-expressing spheroids in three-dimensional (3D) culture as compared to control (Graves et al., 2016, Breast Canc. Res. 18:11). Further, treatment of these podocalyxin-overexpressing MCF7 cells with the myosin inhibitor, blebbistatin, and the small molecule inhibitor of ezrin-actin binding, NSC668394, resulted in decreased collective invasion and migration, respectively. Hence, we hypothesize that podocalyxin, through interactions with the actin cytoskeleton via its cytoplasmic tail binding partners, can facilitate increased collective epithelial tumor cell motility, at least in some contexts. To test this hypothesis, we have generated podocalyxin null MCF7 clones and cell populations.
using CRISPR-Cas9 genome editing and reconstituted these cells with mutant forms of podocalyxin that are unable to interact with the scaffolding proteins NHERF and/or ezrin and hence with the actin cytoskeleton. Preliminary results from 3D culture and live imaging of these mutant podocalyxin-expressing cells suggests that loss of podocalyxin’s cytoplasmic tail results in decreased spheroid invasion that may be a result of deficiencies in acto-myosin contractility. To further elucidate the pathways involved in driving collective tumor invasion that is observed in tumor cells with elevated expression of podocalyxin, we are performing RNA-Seq analysis comparing gene expression in MCF7 cells that lack or overexpress podocalyxin. Together, our data suggest that increased expression and mislocalization of podocalyxin may facilitate aberrant interactions with the actin cytoskeleton and contractile machinery, driving enhanced cell motility and, in certain tumor microenvironments, promote collective tumor invasion.

P161

**The role of circulating cancer associated fibroblasts and circulating tumor stem cells in lung adenocarcinoma.**

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Accumulating evidence suggests that tumor cell behavior is highly influenced by the tumor-microenvironment which plays a determinant role in emergence of circulating tumor stem cells. Within this milieu, cytokines secreted by cancer associated fibroblasts (CAFs) confer a survival advantage to circulating tumor stem cells (CTSCs), these being indicators of residual-disease, by evading the immune system. Collectively, CAFs serve as an "incubator" for CTSCs by providing a so-called favorable "soil" for their subsequent growth in the circulation during EMT. CTSCs may thus be considered as an important target in diagnostic and therapeutic applications. In view of this, using a liquid biopsy approach, this study aimed to develop minimally invasive diagnostic and prognostic modalities for frequent monitoring of treatment response to improve clinical outcomes in lung adenocarcinoma, by generating patient derived pleurospheres (PS) in serum-free media. Here, their chemotherapeutic resistance was evaluated using the MTT assay and EMT status was characterized by western-blot, immunofluorescence and immunocytochemistry. Furthermore, expressions of CSC and epithelial markers were assessed using flowcytometry. cCAFs were cultured in-vitro and characterized by western blot and qRT-PCR using α-SMA marker. PS notably had higher self-renewal potential in serum-free media, suggesting their stem-like characteristics. The MTT assay revealed their intrinsic drug-resistance towards gemcitabine at 100 µM higher concentration, followed by significantly higher expression of MDR markers as disclosed by qRT-PCR. Furthermore, CTSCs showed extensive expression of Vimentin as compared to E-cadherin suggesting occurrence of EMT. Flow cytometry revealed the presence of a higher CD44+(+)/CD24(−) cell population having CK (5.6±0.3%) and EpCAM (0.5±0.4%), suggesting the presence of CTSCs rather than CTCs. Expression of cCAFs at a proteomic level was significantly higher than healthy individuals (p<0.0001). Moreover, qRT-PCR demonstrated all patients were positive for presence of cCAFs; and additionally, a significant trend was observed for cCAFs between different stages of lung cancer patients (p < 0.014), suggesting their probable role in progression. In conclusion, companion biomarkers, cCAFs along with CTSCs, could enhance early detection of lung cancer and could prove to be an efficient biomarker for metastasis as a new paradigm shift in treatment monitoring.
**P162**

**Cell clusters adopt a collective amoeboid mode of migration in confined non-adhesive environments**  
*F. Jaulin; Gustave Roussy Institute, Villejuif, FRANCE.*

Cell migration is essential to most living organisms. Single cell migration involves two distinct mechanisms, either a focal adhesion- and traction-dependent mesenchymal motility or an adhesion-independent but contractility-driven propulsive amoeboid locomotion. Cohesive migration of a group of cells, also called collective cell migration, has been only described as an adhesion- and traction-dependent mode of locomotion where the driving forces are mostly exerted at the front by leader cells. Here, by studying primary cancer specimens and cell lines from colorectal cancer, we demonstrate the existence of a second mode of collective migration which does not require adhesion to the surroundings and relies on a polarised supracellular contractility. Cell clusters confined into non-adhesive microchannels migrate in a rounded morphology, independently of the formation of focal adhesions or protruding leader cells, and lacking internal flow of cells, ruling-out classical traction-driven collective migration. Like single cells migrating in an amoeboid fashion, the clusters display a supracellular actin cortex with myosin II enriched at the rear. Using pharmacological inhibitors and optogenetics, we show that this polarised actomyosin activity powers migration and propels the clusters. This new mode of migration, that we named collective amoeboid, could be enabled by intrinsic or extrinsic neoplastic features to enable the metastatic spread of cancers.

**P163**

**Collective amoeboid migration of tumor cell clusters confined in a non-adhesive environment**  
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Cell migration is essential to most living organisms. Single cell migration involves two distinct mechanisms, either a focal adhesion- and traction-dependent mesenchymal motility or an adhesion-independent but contractility-driven propulsive amoeboid locomotion. Cohesive migration of a group of cells, also called collective cell migration, has only been described as an adhesion- and traction-dependent mode of locomotion where the driving forces are mostly exerted at the front by leader cells. Here, by studying primary cancer specimens and cell lines from colorectal cancer, we demonstrate the existence of a second mode of collective migration which does not require adhesion to the surroundings and relies on a polarized supracellular contractility. Cell clusters confined into non-adhesive microchannels migrate in a rounded morphology, independently of the formation of focal adhesions or protruding leader cells, and lacking internal flow of cells, ruling-out classical traction-driven collective migration. Like single cells migrating in an amoeboid fashion, the clusters display a supracellular actin cortex with myosin II enriched at the rear. Using pharmacological inhibitors and optogenetics, we show that this polarized actomyosin activity powers migration and propels the clusters. This new mode of
migration, that we named collective amoeboid, could be enabled by intrinsic or extrinsic neoplastic features to enable the metastatic spread of cancers.

Monday, December 7, 2020, 12:00 pm

Actin Dynamics in Cell Division

P164

Equatorial non-muscle myosin II and plastin cooperate to align and compact F-actin bundles in the cytokinetic contractile ring

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Cytokinesis is the last step of cell division that physically partitions the mother cell into two daughter cells. It requires the assembly and constriction of a contractile ring, a tightly packed circumferential array of filament actin (F-actin), non-muscle myosin II (myosin) motors and actin binding proteins that forms at the cell equator. Contractile ring assembly has been a topic of extensive study over the years. Even though the mechanism has been identified in fission yeast, how F-actin and myosin accumulate and organize into a circumferential array in animal cells remains to be fully understood. Equatorial compression due to cortical flows and local RhoA-dependent de novo assembly have both been implicated in the process, but whether one of these mechanisms is key or dispensable remains to be clarified. Using C. elegans embryos co-expressing motor dead and wild-type myosin, we show that F-actin cortical flows can be severely reduced without major effects on contractile ring assembly and timely cytokinesis completion. In agreement, fluorescence recovery after photobleaching in the ingressing cleavage furrow reveals that myosin recruitment kinetics is not affected by a significant reduction of actomyosin cortical flows. Importantly, we identify a novel synergy between myosin and the actin filament bundler plastin (PLST-1) in aligning and compacting F-actin bundles at the cell equator. Using live imaging, we measured equatorial F-actin alignment in both single and double perturbations of myosin and PLST-1. In contrast to the single, in the double perturbations F-actin bundles become unstable and completely fail to align, exhibiting oscillating orientations that mostly tend to horizontal rather than the normal vertical alignment. We show that this phenotype is not due to an aggravated cortical flow profile, but to the localized action of both myosin and PLST-1 instead. Altogether, our findings support the idea that contractile ring formation is fundamentally independent of cortical flows and requires the localized and concerted action of myosin and plastin at the cell equator.

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Tropomyosin/actin filaments are required for merging of microtubule asters in mitotic spindle assembly

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An increasing number of studies have demonstrated an important role for the actin cytoskeleton in mitotic spindle assembly in mammalian cells through its interaction with the microtubule network and its dynamic association with spindle poles and the cell cortex during mitotic progression. We have identified a specific population of cortical actin filaments containing tropomyosin Tpm3.1 that is required for the merging of microtubule asters to form a bipolar mitotic spindle via regulating the localization of the NuMA-dynein-dynactin complex to the poles. This mechanism is revealed by exposing HeLa cells to low dose vincristine to induce supernumerary microtubule asters immediately after nuclear envelope breakdown (NEBD). Live-cell imaging was performed to track the behaviour of these microtubule asters in the presence and absence of small molecule inhibitors of Tpm3.1. Many of the asters caused by vincristine single-drug treatment quickly merge with the two centrosome-organized spindle poles; whereas the remainder aggregate into large spindle poles resulting in a multi-polar spindle. Over time the extra acentrosomal poles move towards and combine with the centrosomal poles to form a functional bipolar spindle. The combination of anti-Tpm3.1 compounds with vincristine treatment causes an increase in the number of microtubule asters upon NEBD. Furthermore, the combination disrupts the movement of the asters towards centrosomes, resulting in a persistent multi-polar spindle over a long time period up to 16 h or until cell death. Our fixed-cell imaging results show that those microtubule asters are associated with the NuMA-dynein-dynactin complex, which has been known to cluster microtubules or microtubule asters into spindle poles during early mitosis. Moreover, we found that dynactin and dynein co-immunoprecipitated with Tpm3.1 in mitotic cell extracts, indicating a potential interaction between Tpm3.1 and these motor proteins during mitosis. Taken together, our findings suggest the possibility that microtubule motors are using Tpm3.1-containing actin filaments to facilitate the movement of microtubule asters to centrosomes during bipolar spindle formation.

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The kinesin-like protein Pavarotti functions non-canonically to regulate actin dynamics

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Individual cells within tissues and organs are subjected to damage caused by daily wear and tear and environmental/physiological stresses. To survive this damage and remain functional, cells have a robust repair mechanism comprised of rapid membrane resealing, dynamic cortical cytoskeleton reorganizations, and cytoskeleton/membrane remodeling. In the Drosophila cell wound model, actin and myosin are recruited to wounds and their subsequent assembly into a contractile actomyosin ring is necessary for wound closure. We recently found that three RhoGEFs (RhoGEF2, RhoGEF3, and Pebble (Pbl)) and one RhoGAP (Tumbleweed (Tum)) regulate the spatial and temporal patterns of Rho1, Rac1, and Cdc42, which are major members of Rho family GTPases and indispensable for dynamic actin and myosin regulation. Interestingly, Pbl and Tum are also required during cytokinesis for the formation of a similar actomyosin ring formation, where it activates Rho1 at the equator of two dividing cells. During cytokinesis, Tum associates with Pavarotti (Pav), a kinesin-like protein, to form the centralspindlin complex that moves along microtubules. While Pbl, Tum, and Pav overlap spatially and regulate the
Rho1 activity during cytokinesis, we find that Pbl, Pav, and Tum localization patterns are not identical and Pbl regulates Cdc42 during cell wound repair. Consistent with this, we find distinct phenotypes associated with the depletion of Tum or Pav, suggesting centrosinlin complex-independent roles for these proteins in cell wound repair. When performing drug inhibition and biochemical studies we were surprised to find that the classically microtubule-associated Pav protein binds directly to actin. In addition to cell wound repair, we find that actin-dependent Pav function is required for the normal oogenesis. Our findings have important implications for understanding the biological functions of Pav, as well as the processes of cell wound repair and oogenesis.

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Differential requirements for the Rho GTPases in mitotic and meiotic divisions in echinoderm embryos
D. Pal; New Mexico State University, Las Cruces, NM.

Differential requirements for the Rho GTPases in mitotic and meiotic divisions in echinoderm embryos. Debadrita Pal¹, Andrea Ellis¹, Silvia P. Sepúlveda-Ramírez¹, Torey Salgado¹, Isabella Terrazas¹, Gabriela Reyes, Richard De La Rosa¹, John H. Henson² and Charles B. Shuster¹.¹Department of Biology, New Mexico State University, Las Cruces, NM 88003; and ²Department of Biology, Dickinson College, Carlisle, PA 17013. Rho GTPases are the primary regulators of the actin cytoskeleton in nonmuscle cells, and while Rac and Cdc42 are generally thought to promote viscoelastic, branched actin networks, Rho promotes unbranched actin and actomyosin contractility. Rho organizes the contractile ring during cytokinesis, and while there is consensus that Rac and Cdc42 must be suppressed at the equator, the mechanisms involved remain controversial. Moreover, the respective roles of these proteins are even less clear in atypical divisions such as polar body extrusion during meiosis. To better understand the contributions of Rho GTPase proteins to cell division, we expressed dominant-negative or activated mutants of Rac in sea urchin embryos and sea star oocytes. Live-cell imaging of developing embryos revealed that while Rac was not essential for early mitotic divisions; expression of activated Rac resulted in cytokinesis failure. Expression of activated Rac with effector-binding mutants or in the presence of Arp2/3 inhibitors strongly suggested that activated Rac antagonizes cytokinesis through Arp2/3-mediated actin polymerization. In contrast to mitotic divisions, Rac and Arp2/3 are required for oocyte meiotic divisions in that inhibiting either blocked spindle docking to the cortex, but expression of activated Rac directly suppressed the Rho wave that accompanies the surface contraction and polar body protrusion. Expression of activated Rac with effector binding mutations or in presence of an Arp2/3 inhibitor rescued the Rho wave. Together, these results support the notion that while there may be differential requirements for the Rho GTPases in meiotic and mitotic divisions, the need for a transient suppression of Rac-mediated branched actin filament networks may represent a general mechanism for the promotion of cortical contractility.

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Regulation of actin turnover in the contractile ring by formin, myosin and cofilin during fission yeast cytokinesis
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Cytokinesis, the last step of cell division, physically separates daughter cells from each other. It requires mechanical force provided by the tension-generating actomyosin contractile ring. Continuous turnover of actin filaments is essential for the stability of the contractile ring (Stachowiak et al. 2014) but its mechanism remains unknown. In this study, we examined how formin, myosin II and cofilin contribute to the actin turnover respectively during fission yeast cytokinesis. We employed LifeAct-GFP as the probe to measure the actin turnover with quantitative fluorescence microscopy. We demonstrated that ~200,000 actin molecules assembled in the ring at the start of the constriction in the wild-type cells. The ring constriction was accompanied by a net disassembly of ~6,000 actin molecules/min. A hypomorphic mutant of formin Cdc12p, cdc12-4A, reduced the number of actin molecules in the ring by 50% while the ring constriction was normal. As a result, the net disassembly rate decreased by 50% in this formin mutant. A mutant of the essential type II myosin Myo2p, myo2-E1 reduced the number of actin molecules in the ring by 40% even at the permissive temperature. This myo2 mutation also slowed down the ring constriction and reduced the net disassembly rate of actin by ~70%. Lastly, the hypomorphic mutant of cofilin adf-M3 resulted in highly heterogenous disassembly of actin during the ring constriction in the mutant cells. To better understand the contribution by cofilin, we examined the deletion mutant of Aip1p, which helps cofilin sever actin filaments. The aip1D mutant exhibited reduced, both the number of actin molecules in the ring and the net disassembly rate by 40%. We conclude that formin Cdc12, myosin Myo2 and cofilin each contribute through different mechanism to the turnover of actin filaments in the contractile ring.

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Regulation Of Cell Shape And Mechanics In Dividing Drosophila Stem Cells

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Division is one of the most important events in the life of a cell, which ensures that the genetic material and the entire set of cellular components segregate in the correct way between the two daughter cells. To divide, cells have to undergo profound shape changes in a timely manner. While it is known that these changes are mainly determined by the actin cortex, much remains to be discovered about the molecular and cellular mechanisms that drive partitioning of the cytoplasm and asymmetric cell division. The Arp2/3 complex is a nucleator of actin branched filaments, well-known for its role in the formation of cell protrusion for cell migration, and in intracellular motility of organelles. In this work, we identify the Arp2/3 complex as a potential new regulator of the division process, using Drosophila melanogaster neuronal stem cells as a model system. These are called neuroblasts and are special in that undergo asymmetric divisions. Using different approaches to inhibit the Arp2/3 complex, such as drug treatments and RNA interference, we observed the formation of an ectopic cleavage furrow in dividing neuroblasts. This is a membrane protrusion that appears after the completion of cytokinesis and is marked by ectopic myosin localization. Other phenotypes were also noted - cortical instability phenomena like blebs formation, spindle anomalies (abnormal central spindle and unusually long microtubules) and cell division defects (increased asymmetry between the two daughter cells and longer mitosis). Interestingly, this indicates that although the ectopic cleavage furrow is the most evident result of Arp2/3 inhibition, the other phenotypes suggest a more complex role carried out by the complex during neuroblast division. With the current work we are trying to understand which of the Nucleation Promoting Factors (NPFs) is responsible for regulating Arp2/3 in this context. Since each of them has a different cellular...
localization and it is involved in different pathways, we hope in this way to narrow down the processes in which Arp2/3 is involved. In conclusion, through this work we aim to elucidate new molecular and cellular mechanisms that contribute to asymmetric stem cell divisions in the fly and to shed light on the mechanisms underpinning animal cell division in general.

Adipocytes and Metabolism

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Human subcutaneous adipose tissue expresses catecholamine synthesis pathway genes
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Catecholamines (CA) play key roles regulating metabolism and immunity. Recently CA were demonstrated to be produced by adipocytes underling new functions in obesity. Adipose tissue (AT) catecholaminergic modulation remains a non-appreciated issue in obesity. Tyrosine hydroxylase (TH) and phenylethanolamine N-methyltransferase (PNMT) are essential enzymes for CA biosynthesis. Our model of indirect cell coculture showed that macrophages and adipocytes secretomes co-regulate CA synthesis suggesting a key signaling role for CA connecting immune and metabolic disease clusters (Gomes et al., 2019). Using the dietary-induced obesity C57BL/6 mice model, we have shown TH and PNMT are expressed along preadipocytes differentiation (Gomes et al., 2020). TH and dopamine receptors in peripheral blood mononuclear cells are under expressed in central obesity (Leite et al., 2016), a pattern that can result from inflammation (Leite & Ribeiro, 2020). We propose human AT produces CA and thus investigated TH and PNMT expression along adipocytes differentiation. Human AT was obtained from subcutaneous cellular tissue of abdomen of patients submitted to abdominoplasty. We performed primary adipocyte culture (mature adipocytes) and, afterwards, the stromal vascular fraction was washed and filtered to obtain isolated preadipocytes. Once in confluence, preadipocytes were differentiated into adipocytes. Normalized relative expression (mRNA levels) of TH and PMNT was determined by qRT-PCR in different differentiation days (0, 3, 6 and 10) of human subcutaneous AT. TH and PNMT transcripts are expressed on subcutaneous adipose cells along the differentiation of primary human subcutaneous preadipocytes into adipocytes. The products were run on an agarose gel to confirm amplicon presence. Total AT and the isolated mature adipocytes expressed both TH and PNMT. Our findings show, for the first time, that pre as well as mature adipocytes from human subcutaneous tissue express TH and PNMT mRNA and contamination by CA-producing cells was excluded since we newly differentiated adipocytes originated from preadipocytes primary cultures. Our results open a new and exciting perspective on the way these amines affect AT function, although further studies are needed to fully elucidate their role in obesity-related immunometabolic disease clusters.
**P171**

The heterogeneous nuclear ribonucleoprotein (hnRNP) glorund functions in the *Drosophila* fat body to regulate lipid storage and transport

**A. Kolasa, J. Bhogal, J. DiAngelo; Penn State Berks, Reading, PA.**

The availability of excess nutrients in Western diets has led to the overaccumulation of these nutrients as triglycerides in the body, a condition known as obesity. The full complement of genes important for regulating triglyceride storage and whose alteration leads to obesity is not completely understood. Genome-wide RNAi screens in *Drosophila* cells have identified genes involved in mRNA splicing as important lipid storage regulators. Our lab has shown that a group of splicing factors called heterogeneous nuclear ribonucleoproteins (hnRNPs) regulate lipid metabolism in the fly fat body; however, the identities of all of the hnRNPs that play a role in controlling triglyceride storage are not known. Here, we used the GAL4/UAS system to induce RNAi to the hnRNP glorund (glo) in the *Drosophila* fat body to assess whether this hnRNP has any metabolic functions. Decreasing glo levels resulted in less triglycerides being stored throughout the fly. Interestingly, decreasing fat body glo expression resulted in increased triglyceride storage in each fat body cell, but blunted triglyceride storage in non-fat body tissues, suggesting a defect in lipid transport. Consistent with this hypothesis, the expression of *apolipophorin* (apolpp), *microsomal triglyceride transfer protein* (mtp), and *apolipoprotein lipid transfer particle* (apoltp), apolipoprotein genes important for lipid transport through the fly hemolymph, was decreased in glo-RNAi flies, suggesting that glo regulates the transport of lipids from the fly fat body to surrounding tissues. Together, these results indicate that glorund plays a role in controlling lipid transport and storage and provide additional evidence of the link between gene expression and the regulation of lipid metabolism.

**P172**

*Zw*, the *Drosophila* homolog of G6PD, is a potential 9G8 target to regulate glycogen metabolism in the *Drosophila* fat body

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With the increase in abundance of food in Western cultures, the storage of these excess nutrients can lead to obesity and other metabolic diseases. In animals, excess nutrients from food are stored as triglycerides, mostly as lipid droplets found in adipose tissue. Genome-wide RNAi screens in *Drosophila* cells have identified several groups of genes involved in triglyceride metabolism and storage. One such group of interest to our lab includes RNA splicing factors. Previous studies from our lab have characterized the metabolic roles of a group of splicing factors called SR proteins that function to identify intron/exon borders. One SR protein that we have shown to regulate nutrient storage is 9G8. Decreasing 9G8 function causes an increase in triglyceride and glycogen storage and alters the processing of the lipid breakdown gene, CPT1. To better understand the mechanisms whereby 9G8 causes these changes in triglyceride and glycogen storage, we performed RNA sequencing on flies with decreased 9G8 levels in the adult *Drosophila* fat body. Not surprisingly, differential expression and pathway analysis of the RNA sequencing data showed a down regulation of genes related to various metabolic pathways, including lipid and glucose metabolism. One down regulated gene from this
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dataset involved in glucose metabolism was Zwischenferment (Zw), the *Drosophila* homolog of human glucose 6-phosphate dehydrogenase (G6PD). G6PD regulates the entry of glucose 6-phosphate (G6P) into the pentose phosphate pathway. G6P is also a substrate for glycogen synthesis, so it is possible that decreasing G6P flux into the pentose phosphate pathway would shift flux towards glycogen synthesis. To test this hypothesis, RNAi knockdown of Zw was performed in the adult *Drosophila* fat body. Zw-RNAi flies had an increased glycogen phenotype, similar to that found in flies with RNAi knockdown of 9G8 suggesting a role for Zw in the 9G8-mediated glycogen accumulation phenotype. Together, these findings suggest that 9G8 regulates several metabolic genes and may control the function of Zw to specifically control glycogen storage in the *Drosophila* fat body.

P173

**Characterizing the effects of spermine synthase mutations on polyamine pathway gene expression and macromolecule storage in female *Drosophila melanogaster***

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Polyamines are enzymes integral for proper regulation of cellular proliferation, differentiation, and nucleic acid stability. Altering the expression of enzymes in the polyamine pathway has been linked to developmental abnormalities such as Snyder Robinson Syndrome and variations in lipid metabolism. The goal of this study was to determine the impact of spermine synthase (Sm) on the expression of other polyamine pathway enzymes as well as its influence on lipid metabolism. Utilizing female *Drosophila melanogaster* as a model organism, both a fat body specific knockdown (Yolk-Gal4>Sms-RNAi) and whole-body knockout (Sms<sup>c909</sup>) of Sms were used to determine the effects Sms had on the expression of polyamine pathway enzymes and macromolecule storage. Expression of seven pathway genes, Sms, SpdS, Odc1, Oda, Sat 1/2, PAO, and SamDC, was conducted on Sms<sup>c909</sup> flies. HPLC assessed polyamine content in Sms<sup>c909</sup> flies. Furthermore, triglyceride and glycogen content were analyzed in Sms<sup>c909</sup> flies and fat body specific triglyceride and glycogen storage and DNA content were evaluated in Yolk-Gal4>Sms-RNAi flies. Sms<sup>c909</sup> flies weighed less than wildtype flies and expressed significantly decreased levels of Sms, SpdS, Odc1, and SamDC mRNA and significantly increased levels of Sat1/2 mRNA when compared to wildtype flies. Spermine and spermidine content were notably diminished in Sms<sup>c909</sup> flies, and the ratio of spermidine to spermine was significantly increased when compared to wildtype. Sms<sup>c909</sup> flies showed lower levels of glycogen and triglycerides. Interestingly, in Yolk-Gal4>Sms-RNAi flies only glycogen storage was decreased. Overall, the results suggest that removal of the Sms gene reduces polyamine biosynthesis and interferes with lipid storage. Interestingly, the upregulation of Sat1/2 suggests that polyamine catabolism was induced, perhaps as a mechanism to maintain intracellular polyamine levels. Additionally, the decrease in total triglyceride storage suggests that Sms could affect expression of genes responsible for forming triglycerides such as FASN and ACC, which were previously shown to be affected in ODC mutants. Future experiments could study the expression of lipid metabolic genes in these animals in order to derive a mechanism for lipid metabolism.
**Dietary antioxidant improves hepatic lipid profile in infected and cured of the bacterial infection rainbow trout**

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Bacterial septicemia and other bacterial diseases threaten the health and welfare of farmed fish. Natural food additives, such as antioxidants, prebiotics, or immunostimulants, being used solely or along with the standard therapy with antibiotics, increase fish survival heightening their natural resistance to the infections. The underlying molecular mechanisms of both the infection and dietary additives are poorly understood. In juvenile rainbow trout with diagnosed bacterial septicemia, hepatic signs such as hepatosplenomegaly and hemorrhagic lesions indicated liver pathology and dysfunction were revealed. The lipid profiles of trout liver were analyzed to elucidate the effects of infection, antibiotic therapy, and post-infection recovery in fish fed either a standard or a supplemented with bioflavonoid dihydroquercetin (25 mg per kg of feed) diets. In infected rainbow trout, the physiological accumulation of triacylglycerols, phospholipids, and their essential fatty acid components was suppressed not to reach the normal level even after the antibiotic therapy. The composition of membrane lipids, including sterols, 18:1n-7 fatty acid, and phosphatidylethanolamine, was also abnormal in infected fish and a response was shown to be eliminated by a supplemented diet. In contrast to fish fed a standard diet, n-3 fatty acids prevailed on n-6 polyunsaturated fatty acids in fish fed with a supplement. At the post-infection period, the deficit on polyunsaturated n-6 and n-3 fatty acids in hepatic membrane fraction primarily due to their impaired dietary assimilation was detected in fish that received a standard but not a supplemented diet. In conclusion, substantial impairments in hepatic lipid accumulation and composition induced by the bacterial infection followed by antibiotic treatment were shown to be mitigated by the dietary antioxidant. Apparently, studied natural additive favors well-being and infection resistance in reared fish. The study was supported by the Russian Science Foundation grant no. 17-74-20098.

**Cell-ECM Interactions Governing Cell and Tissue Functions**

**Direct Talin-Actin Binding is Required for Mammalian Development**

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During animal development, cell-extracellular matrix (ECM) adhesion plays an essential role in the assembly and maintenance of complex tissues and organs. The integrin family of transmembrane adhesion receptors are the main links between the ECM and the intracellular actin cytoskeleton, and their functions are carefully regulated to ensure proper tissue development and homeostasis. Talin, which is a cytoplasmic protein that links integrins to the actin, is known to act as a molecular scaffold for the assembly of the integrin adhesion complex and it is extremely important for mammalian development. Talin has three actin binding sites (ABS) and the ABS3 site located at the C-terminal end of talin is thought to be essential for the integrin-mediated mechanical signaling. Specifically, it has been
proposed that when integrin bound talin links to actin through ABS3, it allows the mechanical force to be transduced to the cytoplasmic adhesion complex. Such force transduction stretches talin and exposes its cryptic binding sites that recruit additional components like vinculin. This can promote more cytoskeleton interactions and thus further reinforcing the integrin-mediated cell-ECM adhesion. To explore the role of ABS3 in vivo, we generated mice containing the K2443D/V2444D/K2445D (KVK/DDD) mutation in Tln1 that abolishes actin binding through ABS3. From heterozygote intercrosses, we have observed that Tln1<sup>KVK/DDD</sup> mutants showed developmental defects and reduced sizes starting at Embryonic day (E)8.5 and became resorbed around E11.5. We will present phenotypic characterization of Tln1<sup>KVK/DDD</sup> mutants as well as analysis of cellular and subcellular phenotypes associated with the Tln1<sup>KVK/DDD</sup> mutation in primary fibroblasts derived from the mouse embryos. In summary, our data have supported the idea that direct talin-actin binding plays important roles during early mouse development.

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The Rho-GEF PIX pathway directs assembly of lateral attachment structures between striated muscle cells

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The adhesion of cells to extracellular matrix (ECM) is facilitated by “integrin adhesion complexes” (IACs), consisting of integrin and many associated proteins. Although much is known about the composition and initial assembly of IACs, we do not know what determines where an IAC will form. Myofibrils in muscle cells are connected to the cell membrane via costameres, muscle-specific IACs. <i>C. elegans</i> striated muscle has IACs at the M-line, dense body, and attachment plaque at muscle cell boundaries (MCBs). From a screen for mutants that disrupt the localization of IAC component PAT-6 (α-parvin), we discovered that loss of function of <i>pix-1</i> results in the absence of PAT-6, and other IAC components, only at MCBs. PIX-1 is the nematode ortholog of mammalian β-PIX, and contains SH3 and RhoGEF domains. Muscle-specific expression of wildtype PIX-1 rescues the MCB phenotype. In addition to deficiency of PIX-1, overexpression of wild type PIX-1 protein results in decreased locomotion and disrupted MCBs. PIX-1 localizes to all 3 IAC structures. Based on studies of β-PIX and of PIX-1 in other tissues in <i>C. elegans</i>, we hypothesize that in muscle, PIX-1 activates Rac or Cdc42 via the scaffold GIT-1, and this GTPase acts through a PAK protein kinase to phosphorylate key substrates. Loss of function mutants in each of the known pathway proteins result in disrupted MCBs. Worms carrying CRISPR/Cas9 mutations in PAK-1 that make the kinase either constitutively active or catalytically dead, show disrupted MCBs, demonstrating that increased or decreased kinase activity yields the same phenotype. A <i>pix-1</i> null mutant and a <i>pix-1</i> missense mutant in a highly conserved residue in the RacGEF domain show ~50% reduction in the level of activated (GTP bound) Rac in muscle. Rho GTPases function as molecular switches between active and inactive states, facilitated by GEF and GAP proteins, respectively. However, a GAP for PIX-1 in nematodes or β-PIX in mammals has yet to be identified. In <i>C. elegans</i>, there are 30 proteins containing RhoGAP domains, and 18 of them are expressed in muscle. Upon screening mutants of all 18 genes, RRC-1 and HUM-7 mutant alleles yield the same MCB defect as <i>pix-1</i> mutants. Therefore, our results provide the first evidence that the PIX pathway has an important function in muscle, and the first to identify two candidate GAP proteins for the PIX pathway.
Coupling to substrate adhesions by talin, focal adhesion kinase, vinculin and DIAPH1 drives the maturation of muscle stress fibers into myofibrils within cardiomyocytes

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Forces generated by heart muscle contraction must be balanced by adhesion to the extracellular matrix (ECM) and to other cells for proper heart function. Decades of data have suggested that cell-ECM adhesions are important for sarcomere assembly. However, the relationship between cell-ECM adhesions and sarcomeres assembling de novo remains untested. Sarcomeres arise from muscle stress fibers (MSFs) that are translocating on the top (dorsal) surface of cultured cardiomyocytes. Using an array of tools to modulate cell-ECM adhesion, we established a strong positive correlation between the extent of cell-ECM adhesion and sarcomere assembly. Specifically, we show that decreased cell-ECM adhesion by knockdown of Vinculin, DIAPH1, or focal adhesion kinase, or the expression of a dominant negative talin construct, resulted in attenuation of myofibril maturation. We also show that increased cell-ECM adhesion by either increasing the concentration of ECM or by inhibiting the kinase activity of focal adhesion kinase resulted in precocious myofibril maturation. On the other hand, we found a strong negative correlation between the extent of cell-ECM adhesion and the rate of MSF translocation, a phenomenon also observed in nonmuscle cells. We further find a conserved network architecture that also exists in nonmuscle cells. Taken together, our results show that cell-ECM adhesions mediate coupling between the substrate and MSFs through vinculin, DIAPH1, talin, and focal adhesion kinase, allowing their maturation into sarcomere-containing myofibrils.

Mechanical microenvironment regulates epithelial defence against cancer via force-sensitive protein localization

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Epithelial cells sustain the tissue form and function by maintaining a tightly-regulated force equilibrium with its mechanical microenvironment. Cancer-associated stiffening of this microenvironment promotes metastasis during advanced malignant stage. Yet, it remains elusive how an existing pathologically stiffened microenvironment might affect the initial pre-malignant stage. At this stage, normal cells usually eliminate the transformed cells by competition, providing a basic epithelial defence against cancer. Here we report that a quantitative increase in the mechanical stiffness of extracellular matrix beyond a threshold value leads to a qualitative phase transition-like degradation of epithelial defence against constitutively active HRas oncoprotein. At multicellular-level, matrix stiffening attenuates the mechanical imbalance between normal and transformed populations. At molecular-level, it sequesters an actin cross-linker protein, filamin, to perinuclear cytoskeleton, making filamin unavailable for epithelial defence. Perinuclear localization of filamin depends on the mechanically tensed cytoskeleton-nucleoskeleton coupling. Disrupting this coupling restores competitive elimination of transformed cells on stiff matrix. Taken together, these findings provide multi-scale explanations answering how tissue...
stiffening due to hyperactive wound healing, fibrosis, or ageing might predispose the epithelium to high cancer risk.

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**Tensin1 contributes to focal adhesion disassembly at mitosis to relieve an integrin-inactivation G2-M checkpoint.**

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Cell rounding at mitosis is required for proper spindle positioning, orientation and stability, and thus is critical to correct chromosome segregation. For a dividing cell to round up, it must reduce its contact area with the extracellular matrix (ECM). This can be achieved by increasing cell cortical tension beyond the cell-ECM adhesion strength, reducing cell-ECM adhesion, or both. Here, we tested the hypothesis that mitotic cell rounding may be mediated by reducing cell-ECM adhesion through focal adhesion (FA) disassembly. Using live cell imaging of FA and cell cycle markers, we found that FA loss is a prophase event that starts before nuclear envelope breakdown and coincides with cyclin B1 translocation to the nucleus. Analysis of FA dynamics showed that during mitosis, the number of assembling FA decreased while the FA disassembly rate increased. Traction force microscopy showed that loss of cell adhesion was accompanied by a rapid drop in traction forces right at the onset of cell detachment with no apparent pre-de-adhesion peak, suggesting that FAs are not disassembled simply by increasing cell contraction beyond the adhesion strength, but undergo a regulated disassembly. Inhibition of mitotic kinases showed that at mitotic onset, PLK1 activity was required for stopping FA assembly, while FA disassembly required CDK1 activation. To examine the possible pathways governing FA disassembly downstream of CDK1, we manipulated regulators of FA disassembly known from studies of cell migration, including myosin II, FAK, calpain and integrins. This showed that neither calpain, myosin II nor FAK activity were required for cell de-adhesion during mitosis, suggesting that FA disassembly at mitosis requires a cell cycle-specific mechanism. Accordingly, we found that the integrin-actin linker tensin 1 exhibit mitotic-specific post-translational modifications yet to be determined. Knocking down tensin1 using siRNA lead to a delay in mitotic entry and a decrease in rounding time suggesting a role in cell de-adhesion during G2/M. Locking integrins in their active/high affinity ECM-binding conformation using either manganese or conformation-specific antibodies showed that integrin inactivation was required for mitotic deadhesion. Unexpectedly, blocking integrin inactivation in cells expressing PCNA as a marker of cell cycle stage inhibited mitotic entry, with the PCNA localization pattern indicating a G2 block. Taken together, our data suggest that mitotic cells use a specific mechanism, distinct from the ones used by migrating cells but dependent on Cdk1 and Plk1 activity, to de-adhere at mitosis. Our data further suggest that integrin inactivation and FA disassembly are required for cell cycle progression in adherent cells.
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**Active sorting of contracting blood clots**  

Blood clots/thrombi undergo volume shrinkage (contraction) driven by platelets. Contraction changes the ability of thrombi to impede blood flow, withstand deformations, and lysis. We show that during contraction fibrin and platelets segregate to the periphery, while RBCs are compressed into the clot’s core. To reveal the physical mechanisms driving this segregation, a mechano-chemical model that relates the feedback between platelet contractility and the dynamic stiffness of the clot was developed. Our model predicts that as resistance to contraction of platelets increases, their mechanosensitive pathways are upregulated and the active free energy decreases. Redistribution lowers the system’s free energy. This mechanism of increasing contractility in response to substrate stiffness provides a basis for the kinetics of segregation as a function of clot composition and platelet contraction. Our model predicts the reduction in clot contraction observed in thrombotic conditions. These findings provide a physical basis for this novel biological active-sorting phenomenon.

P181

**Mitofusin-2 regulates leukocyte adhesion through the maturation of β2 integrin activation in differentiation**  
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Neutrophils are critical in inflammation and innate immunity. Neutrophil adhesion to vascular endothelium is a crucial step in neutrophil recruitment. Mitofusin-2 is required for neutrophil adhesion, but molecular details are unclear. Here, we demonstrated that β2-integrin-mediated slow-rolling and arrest, but not PSGL-1-mediated cell rolling, are defective in mitofusin-2-deficient neutrophil-like HL60 cells. This adhesion defect is associated with reduced expression of fMLP receptors and β2 integrins as well as the inhibited β2 integrin activation, as assessed by conformational-specific antibodies. Mitofusin-2 deficiency limited the maturation of β2 integrin activation during the neutrophil-directed differentiation of HL60 cells, which is indicated by identified markers CD35 and CD87. Reversed to results in overall HL60 cells, mitofusin-2 knockdown in β2-integrin-activation-matured cells (CD87high population) enhanced integrin activation. Our study illustrates the function of mitofusin-2 in leukocyte adhesion and may provide new insights into the development and treatment of mitofusin-2-deficiency-related disease.
Chemotaxis

P182

Chemotaxis in uncertain environments: hedging bets with multiple receptor types
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Eukaryotic cells are able to sense chemical gradients over a wide range of environments, sometimes expressing multiple receptor types with different affinity for the same signal. When do cells gain additional information about a chemoattractant from multiple receptor types, and how can they integrate this information efficiently? We use mathematical modeling to show that, if a cell is exposed to a widely variable environment, it may gain chemotactic accuracy by expressing receptors with varying affinities for the same signal. As the uncertainty of the environment is increased, there is a transition between cells preferring to express a single receptor type and a mixture of types -- hedging their bets against the possibility of an unfavorable environment. We use this to predict the optimal receptor dissociation constants given a particular environment. In sensing this signal, cells may also make multiple measurements over time. Surprisingly, time-integration with multiple receptor types is qualitatively different from gradient sensing by a single receptor type -- it is possible for cells to extract orders of magnitude more information out of a signal than expected by naive time integration. These results may explain in part why Dictyostelium expresses different combinations of cAMP receptors throughout its development.

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Retrograde Localization of the Contractile Vacuole is Critical for Signal Relay and Cellular Streaming in Dictyostelium discoideum
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Distinct changes often occur in morphology during the formation of cellular polarity. In the case of directed migration, cells often form a defined front and rear, as they migrate towards or away from migratory cues. In many cell types, the microtubule organizing center and nucleus take up discrete localizations relative to one another. During the early process in fruiting body development, Dictyostelium discoideum cells polarize and follow one another in an organized manner. We found that the contractile vacuole (CV) network also polarizes towards the rear of the cell, largely co-localizing with the microtubule network. Observations with light microscopy of retrograde CV movement were confirmed by imaging cells expressing the fluorescently tagged CV marker. CV diastole is coordinated with propagating cAMP waves during cell streaming. The cAMP transporter AbcC8 localizes to the CV network. Interestingly, a correlation was observed between the cAMP signal relay, systole, and oscillatory cell movements. The condition that manipulates the CV cycle result in CV cycle inhibition and the timing of loss and gain of the CV cycle mirrored the loss of cAMP signal relay. Mutants lacking the huntingtin protein (htt') lacked a detectable CV in the vegetative state, had smaller than averaged sized CVs when polarized, made very weak cAMP waves and didn't stream. The CV was absent from areas containing F-actin based protrusions and PI3 Kinase activity and localized to areas where the tumor suppressor PTEN was active throughout the life cycle, independent of the actin and microtubule.
cytoskeleton. Our results demonstrate that the CV is part of the polarity network and its posterior redistribution is critical for recruiting cells into streams and is a major regulator of cAMP secretion/relay

P184

Engineering Leukocytes for Enhanced Pathogen Hunting
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The immune system plays a central role in how the body responds and recovers from infectious agents, cancer, and autoimmune disease. Current therapeutics for these diseases mainly rely on pharmacological interventions, often leaving the patient ill or with altered physiology. However, with the rise of Chimeric antigen receptor T cells (CAR T-cells), developing engineered leukocytes to combat disease has become a reality. To exert immunological functions, leukocytes must migrate to the site of disease or infection. This directed cell migration requires the cell to perform directional sensing, form a front and back (polarization), and sense and respond to mechanical stress (mechanoresponsiveness). Chemotactic leukocytes often have phosphoinositide signaling at the anterior membrane and myosin filament enrichment at the posterior cortex. In the presence of chemoattractants, phosphoinositide-3-kinase (PI3K) is translocated to the anterior membrane to stimulate PIP₃ production, leading to the recruitment and activation of Pleckstrin homology (PH) domain proteins, such as Akt. Such phosphoinositide signaling results in cell polarization. Non-muscle myosin IIB (NMIIB) assembles into bipolar filaments and generates contractile stress at the cell rear to help drive migration. Previously, our lab found that altered basal assembly of NMIIB tunes NMIIB mechanoresponsiveness in a biphasic manner and can be regulated by phosphorylation of the myosin heavy chain. Hypothesizing that NMIIB mechanoresponsiveness can be tuned for optimal response in leukocytes, we aim to combine front and back controllers to obtain enhanced chemotaxis. To synthetically enhance the regulatory elements at the front of the cell, we developed a sensor-actuator system containing the iSH₂ domain of PI3K and the PH domain of Akt. In this system, the PH domain senses and binds to PIP₃ at the membrane, and the iSH₂ domain recruits the catalytic p110 subunit of PI3K, forming a synthetic positive feedback. Expression of the iSH₂-PHAkt sensor-actuator in leukocytes resulted in a two-fold increased migratory speed and enhanced chemotaxis, likely due to enhanced polarization. To enhance contraction at the rear of migrating leukocytes, we exogenously express wild type (WT) and mutant NMIIBs in a leukocyte model system with no detectable endogenous NMIIB. We found that exogenous expression of WT NMIIB or mutant forms resulted in a range of motility phenotypes, likely due to changes in cortical tension and adhesion. We are engineering the iSH₂-PHAkt front controller and NMIIB back controller under a pathogen inducible promoter with the goal of coupling these controllers to achieve optimized chemotaxis in the presence of pathogens, developing a self-regulatory and patient friendly therapeutic strategy.

P185

Membrane shaping proteins control lamellipodia dynamics and cell migration
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Cell motility has a critical role in a range of biological processes including development, immunity and disease. Navigation through complex and ever-changing environments often relies on the activity of actin-rich protrusions at the leading edge, also referred to as lamellipodia. Its dynamics determines motion persistence, which is a key parameter for directed cell migration. Lamellipodia are known to exhibit areas of continuously rearranging membrane curvature. However, whether and how curvature-sensitive membrane shaping proteins contribute to leading edge dynamics and function, remains poorly understood. Neutrophils display one of the most dynamic and prominent lamellipodia among all vertebrate cells, and are emerging as a key model system to investigate the role of membrane curvature in the context of cell migration. We followed the transition of neutrophil-like cells from a blast into a differentiated, lamellipodia-forming state using RNAseq, and identified the most highly enriched curvature-sensitive membrane remodeling proteins in this process. After probing their localization to assess enrichment at the leading edge, we generated CRISPR/Cas9 knockout cell lines of promising candidates. We quantitatively assessed changes in cell shape, membrane tension and actin polymerization, focusing on their contribution to motility in various types of environments. Using a combination of machine learning-based segmentation for time-resolved TIRF microscopy and microfluidics, we reveal that motion persistence and directionality, in both freely moving and environmentally constrained cells, strongly depend on the activity of particular BAR domain proteins. Specifically, we show that such proteins control lamellipodia dynamics by regulating WAVE2-driven actin polymerization. KO cells migrate faster and are more persistent during unobstructed migration, but fall short when a change in direction is required. In summary, we show that curvature-sensitive and membrane remodeling BAR domain proteins are key to direct cell motility in complex environments by controlling lamellipodia dynamics.

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Shootin1b as a clutch molecule for dendritic cell chemotaxis
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Dendritic cells are immune cells that migrate toward chemokines such as CCL19; chemotaxis play critical roles during their migration. Actin filaments, powered by their polymerization and myosin II activity, undergo retrograde flow at the leading edge of migrating cells. It has been proposed that the coupling between F-actin retrograde flow and cell adhesion by a clutch molecule transmits the force of F-actin retrograde flow to the adhesive substrate, thereby producing the traction force for cell migration. However, it is unclear whether dendritic cells utilize clutch molecules for their migration. We previously reported that shootin1a functions as a clutch molecule that couples F-actin retrograde flow and the cell adhesion molecule L1-CAM for axon outgrowth and netrin-1-induced axonal chemotaxis (Shimada et al., J. Cell. Biol. 2008; Kubo et al., J. Cell. Biol. 2015; Baba et al., eLife. 2018). We also found that shootin1b, a splicing variant of shootin1a, is expressed in dendritic cells (Higashiguchi et al., Cell Tissue Res. 2016). Here, we report that shootin1b couples F-actin retrograde flow and the cell adhesion molecule L1-CAM in dendritic cells. A chemokine CCL19 promoted PAK1-mediated shootin1b phosphorylation; this shootin1b phosphorylation, in turn, promoted shootin1b interaction with the actin binding protein cortactin and L1-CAM. Furthermore, the migration speed of dendritic cells toward CCL19 source was
decreased by shootin1 knockout. These data suggest that shootin1b functions as a clutch molecule for CCL19-induced chemotactic migration of dendritic cells.

**P187**

**Lymphocyte Egress Signal Sphingosine-1-Phosphate Promotes ERM-Guided, Bleb-Based Migration**

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Ezrin, Radixin, and Moesin (ERM) family proteins form a regulatable linkage between the actin cortex and the underlying plasma membrane. Mutations in ERM proteins cause severe combined immunodeficiency in humans, but the physiological role of these proteins in lymphocytes remains poorly understood. By utilizing mice in which T cells are devoid of all ERM proteins, we find that these proteins are selectively critical for S1P-induced egress from lymphoid organs. ERM-deficient T cells migrate poorly towards S1P \textit{in vitro}, despite normal S1P-induced signaling and receptor expression. By contrast, migratory responses to standard protein chemokines occur normally in the absence of ERM proteins. We found that this selective defect occurs because S1P promotes a fundamentally different mode of migration than chemokines, one characterized by heightened intracellular pressure and bleb-based motility. In this context, ERM proteins facilitate productive, directional migration by limiting blebbing to the leading edge. We hypothesize that the distinct modes of motility elicited by S1P and chemokines are specialized to permit passage across lymphatic barriers and through tissue stroma, respectively.

**Chromatin and Chromosome Organization**

**P188**

**Toward understanding the dynamic state of 3D genome by polymer modeling of Hi-C data**

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The three-dimensional (3D) genome organization and its role in biological activities have been investigated for over a decade in the field of cell biology. Recent studies using live-imaging and polymer simulation have suggested that the higher-order chromatin structures are dynamic; the stochastic fluctuations of nucleosomes and genomic loci cannot be captured by bulk-based chromosome conformation capture techniques (Hi-C). In this work, we focus on the physical nature of the 3D genome architecture. We first describe how to decode bulk Hi-C data with polymer modeling. We then introduce our recently developed PHI-C method, a computational tool for modeling the fluctuations of the 3D genome organization in the presence of stochastic thermal noise. We also present another new method that analyzes the dynamic rheology property (represented as microrheology spectra) as a measure of the flexibility and rigidity of genomic regions over time. By applying these methods to real Hi-C data, we highlighted a temporal hierarchy embedded in the 3D genome organization; chromatin interaction boundaries are more rigid than the boundary interior, while functional domains emerge as dynamic
fluctuations within a particular time interval. Our methods may bridge the gap between live-cell imaging and Hi-C data and elucidate the nature of the dynamic 3D genome organization.

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Cryo-EM structures of nucleosomes in interphase and metaphase chromosomes
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Chromatin and nucleosome structure regulate multitudes of biological reactions on the genomic DNA, such as mitotic chromosome formation, chromosome segregation, transcription, and replication. While near-atomic resolution structures of the mono- and poly- nucleosomes have been solved using in vitro reconstituted nucleosomes with recombinant proteins, diversity, and cell cycle-dependent regulation of nucleosomes in vivo remain unknown. We established a method to isolate nucleosomes from physiologically functional interphase and metaphase chromosomes assembled in Xenopus egg extracts while preserving intact protein-DNA structures that can be analyzed by cryo-EM. With this method, we determined the structures of the nucleosome (~3.4 Å) and the linker histone H1.8-nucleosome complex (4.4 Å) isolated from interphase and metaphase chromosomes, as well as other cofractionated macromolecules, such as actin and lectin. Regardless of nonuniform DNA sequences and cell cycle stages, core parts of interphase and metaphase nucleosomes are identical to known crystal structures. Local resolution analysis of nucleosome EM structures implies that DNA positioning is selectively stabilized at SHL 0±1 and ±4. Two cell cycle-dependent structural changes are observed, however. First, DNA positioning is less stabilized in interphase nucleosomes than in metaphase chromosomes. Second, the structure of linker histone H1.8, which stabilizes two linker DNAs, is solved exclusively on the dyad position of metaphase nucleosomes. However, linker DNA angles are closed in most interphase and metaphase chromatin even without detectable H1. These data indicate that linker angles are not determinant of chromatin compaction status. Altogether, this study illustrates the extraordinary uniform structure of nucleosomes in vivo.

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Learning the distribution of single-cell chromosome conformations in bacteria reveals emergent order across genomic scales
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The spatial order and variability of chromosomes is important to guide cellular functions such as transcription and segregation. Such organizational information is contained within the distribution of chromosome conformations, which remains elusive. To elucidate this, we develop a fully data-driven maximum entropy approach to extract single-cell 3D chromosome conformations from normalized Hi-C experiments, and apply this to the model organism Caulobacter crescentus. This model provides insight into single-cell variability and population averages, revealing which chromosomal features are ordered. We find that on large genomic scales, chromosomal loci exhibit striking long-ranged two-point correlations, indicating emergent order. On smaller genomic scales, our model reveals a pattern of local chromosome extensions that partially correlates with transcriptional and loop extrusion activity. Finally,
we introduce the concept of chromosomal localization information, quantifying the information contained in chromosome organization that may guide cellular processes. Our approach can be extended to other species, providing a general strategy to resolve variability in chromosomal organization at the single-cell level.

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_Drosophila Speciation Core Complex integrity is necessary for pericentromeric localization and hybrid lethality_

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The rise of new species is a keystone in the formation and maintenance of biodiversity. Species-specific adaptations to the environment result from underlying species-specific networksof molecular interactions. In turn hybridization of different species resultsin the breakdown of such finely tuned molecular mechanisms and typically in the lethality or infertility of the hybrids. Because of its fast evolving nature,heterochromatin around centromeres constitutes a strong driver of speciesadaptation and divergence. In response to their changing environment,heterochromatin-binding proteins must evolve accordingly leading to an evolutionary molecular arms race. In the sibling species _Drosophilamelanogaster and Drosophila simulans_ the two heterochromatic proteinsHMR and LHR interact together and are responsible for hybrids lethality. A critical step to understand how these two species have diverged to evolve theirspecies-specific molecular networks is to characterize the nature of their interaction and the protein complex they belong to. Here we use co-immunoprecipitation coupled with Mass Spectrometry in _D. melanogaster_ to define a Speciation Core Complex (SCC) including four subunits in addition toHMR and LHR. We then used HMR mutants to dissect the SCC and show that the integrity of the complex is necessary for both peri-centromeric localization and hybrid lethality.

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Chromatin mobility during DNA repair

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Nuclear DNA of eukaryotes is compactly packaged with histone proteins to form chromatin. Chromatin organization and the constant motion of the DNA may regulate genomic functions like gene expressions, DNA synthesis, and DNA repair. Two major pathways are used for repair of DNA double-strand breaks (DSB): non-homologous end joining (NHEJ) and homologous recombination (HR). The reciprocal dependencies of repair by HR and NHEJ and chromatin mobility are largely unknown in higher eukaryotes. We are characterizing these dependencies using osteosarcoma cells coexpressing histone H2A tagged with photoactivatable GFP (PAGFP-H2A) and a DNA damage sensor (mCherry-53BP1). Cells are treated with DSB-causing agents like mitomycin C and bleomycin after a specific inhibition of either DSB repair pathway by a RAD51 inhibitor (HR) or a DNA-PK inhibitor (NHEJ). In addition, we use siRNA-based inhibition of HR and NHEJ as a complementary approach to manipulate DSB repair pathway usage. As HR repair is mostly restricted to the S phase of the cell cycle, cells in S phase are selected via live cell cycle analysis using fluorescent nucleotides and a NTP transporter system for rapid labeling of DNA. Motions
of chromatin microdomains (PAGFP-H2A) are captured using structured illumination with a diffractive optical element. The system produces a 7x7 array of photoactivated chromatin spots. Motions of the 49 spots are captured with time-lapse imaging and particle tracking and related to DNA damage presence (mCh-53BP1). Here, we present validation of the approach and preliminary results on chromatin dynamics in HR vs. NHEJ repair. In conclusion, the relationship between chromatin mobility and DSB repair pathways is being studied to understand the regulatory mechanism and the tempo of chromatin motions.

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**Synthetic regulatory genomics of mammalian Hox clusters**

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The coordinated expression of Hox genes is crucial for embryonic patterning. In mammals, Hox genes are organized into compact clusters that are under strong purifying selection. The action of local transcription factor binding and distal enhancer elements have emerged as the two major regulatory modes controlling the initiation and maintenance of Hox gene expression. However, a synergistic model describing the relative contribution of these locus intrinsic and extrinsic modes has remained elusive, which will require the ability to manipulate them in concert. Despite the emergence of CRISPR/Cas9 based genome editing, there is a gap in our ability to make multiple, precise edits on the same haplotype or generate large scale complex rearrangements to interrogate the multi-way functional connections between regulatory elements. In the absence of such tools, there has been a focus on defining individual cis regulatory elements that are necessary in the endogenous context to control gene expression. To address this limitation, we developed a ‘regulatory reconstitution’ approach that enabled us to identify a minimal set of elements that were sufficient, and not just necessary, to specify the dynamic regulation of HoxA genes. Bottom-up synthesis of large DNA segments (>100kb) allows for the arbitrary modification, removal and inclusion of elements on the scale that is required to probe gene regulation. We coupled the bottom-up synthesis of mammalian HoxA clusters (130-170kb) containing subsets of locus constituents, with their site-specific delivery to an ectopic location in the mouse genome. We analyzed the behavior of these synthetic, ectopic Hox clusters in an in vitro differentiation system through epigenomic and transcriptomic analyses. We find that a minimal Hox cluster is sufficient to specify and establish stable chromatin domains in response to developmental patterning signals, independent of enhancers. Enhancers serve to increase both total transcriptional output from the clusters and to improve the resolution of the chromatin boundary. Thus, our data suggest that compact Hox clusters contain all the information to decode and maintain positional information. We expect this to be a proof-of-principle for ‘Synthetic Regulatory Genomics’ - a broadly generalizable approach for the study of gene regulation in complex genomes.

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**Arima- HiC technologies for the comprehensive analysis of chromatin conformation’s impact on gene regulation and for the phased de novo assembly and variant detection of large genomes**

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Chromosome conformation capture technologies developed by Arima Genomics, such as Arima-HiC+, Arima-HiChIP, and Arima Capture-HiC, are powerful approaches for profiling 3D genome structure and providing valuable insights into the mechanisms of gene regulation in human disease. Disease-specific chromosome folding has been implicated across numerous human pathologies, such as cancer and have been valuable for the functional interpretation of non-coding disease associated variants (GWAS). The ability to physically connect distant regions of the same chromosome without the need to isolate ultra-long DNA molecules can also be leveraged for applications such as the phased de-novo assembly of diploid genomes, the validation and phasing of assembled contigs and the discovery and phasing of single nucleotide, InDel and structural variants. Arima’s R&D efforts to improve coverage uniformity have resulted in the broad utilization of Arima sample prep solutions across various assembly projects, like the Vertebrate Genome Project and the Darwin Tree of Life. We will showcase the utility of high-coverage Arima-HiC data for chromosome-scale scaffolding of vertebrate genomes and report on our sample collection and preservation methodologies. We will also highlight the results of a recent collaborative publication that demonstrated utility of our technology towards haplotype-resolved chromosome-scale de novo assembly of 3 human samples PGP1, HG002 and NA12878 with contig NG50 of up to 25 Mb and scaffold NG50 of up to 130 Mb. Around 99.5% of the heterozygous loci could be phased to over 98% accuracy, outperforming other approaches in terms of both contiguity and phasing completeness. Our reproducible and flexible kit platform covering genome-wide (Hi-C) and targeted (HiChIP, Capture-HiC) genome structures is based on our core proximity ligation chemistry. The 6-hour protocol improves analytical sensitivity through rapid, multiple restriction enzyme Hi-C chemistry. This improved technology detects more chromatin folding features from significantly reduced sequencing depth. Arima-HiC kits have been validated through scientific publications that cover oncology, cardiology, neurobiology, and immunology. The targeted chromosome conformation capture technologies from Arima Genomics enhance the ability of clinical and translational researchers to study pathological mis regulation of chromosome conformation in fine detail and at reduced costs. We will present our customer validated H3K27ac and H3K4me3 HiChIP protocols, demonstrating reproducible detection of long-range interactions at active promoters as well as data from high-resolution (500bp) Capture-HiC experiments targeting oncogenes and tumor suppressors in a panel of 10 cancer and non-cancer samples.

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**Linker histone regulates mitotic compaction through condensin I and TopoIIα**

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DNA loop extrusion by condensin and decatenation by DNA topoisomerase II (Topo II) drive mitotic chromosome compaction and individualization, but it is unclear how these enzymes act together on chromatin. Here, we demonstrate that the linker histone H1.8 suppresses chromatin levels of both condensins and Topo II through competitive inhibition. Chromosome morphology and Hi-C analyses suggest that H1.8-mediated suppression of condensin I shortens mitotic chromosome lengths by decreasing DNA loop numbers while increasing loop sizes. We also show that Topo II activity, which is enhanced by condensin I, is suppressed by H1.8 during mitosis, and that this suppression of condensin
and Topo II by H1.8 limits chromosome individualization. Accordingly, depletion of H1.8 compromises spindle integrity with hyper-individualized, elongated chromosomes. While linker histones locally compact DNA by clustering nucleosomes, our study reveals the mechanism by which H1.8 controls megabase-scale DNA folding and topological organization through repressing condensin I and Topo II activities.

**Chromosome Organization: Model Organisms**

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**The Synaptonemal Complex mediates chromosome alignment by balancing dimerization and aggregation**
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During sexual reproduction the parental homologous chromosomes find each other and align along their lengths. Chromosome pairing and alignment integrate local sequence homology with large-scale contiguity, thereby allowing for precise exchange of genetic information while preserving genome integrity. The Synaptonemal Complex (SC) is a conserved phase-separated interface that assembles between the homologous chromosomes, forming a zipper-like structure that brings chromosomes together and regulates exchanges between them. Here we isolated and characterized two mutations in the dimerization interface at the middle of the SC zipper in *C. elegans*. The mutations perturb both chromosome alignment and the regulation of exchanges. Underlying the chromosome-scale phenotypes are distinct alterations to the way SC subunits interact with one another. We propose that the SC brings homologous chromosomes together by balancing two biophysical properties: obligate dimerization that prevents the SC from assembling on unpaired chromosomes; and a tendency to self-interact and phase-separate, allowing pairing interactions to extend and span entire chromosomes.

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**Investigating chromosome-specific biology during meiosis**
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Chromosomal aneuploidy in oocytes is one of the leading causes of infertility and miscarriage in women. Interestingly, aneuploidy rates vary between chromosomes, suggesting that each chromosome may have unique underlying biology and distinct behaviors during meiosis. Aneuploidy is the result of chromosome missegregation during meiosis and can occur at an elevated frequency when wild-type recombination patterns are altered. However, the direct contribution of the meiotic machinery to chromosome-specific recombination patterns and aneuploidy remains unknown. One of the most important components of the meiotic machinery is the synaptonemal complex (SC), a conserved meiotic protein structure that regulates the repair of programmed double-strand breaks (DSBs) into either crossovers or non-crossover gene conversions. We used CRISPR/Cas9 to generate three distinct targeted in-frame deletion mutations within the same SC protein in *Drosophila*. These mutations caused
premature SC breakdown in a range of meiotic stages (early pachytene, early-mid pachytene, and mid pachytene), allowing us to directly assess the importance of intact SC throughout early meiosis. With this temporal resolution, we investigated the requirement for full-length SC throughout meiosis, including clustering of centromeres, chromosome pairing, and recombination on a chromosome-specific level. As the SC fragments, we found that the X chromosome displays a loss of a pairing and a near complete loss of recombination, retaining only infrequent centromere-proximal crossovers. In contrast, the autosomes maintain centromere-proximal pairing and have increased proximal recombination compared to wild type. We used next-generation sequencing of progeny from our SC mutants to demonstrate that non-crossover gene conversion events occur at similar rates on the autosomes and still occur on the X chromosome, including in regions where crossovers are highly suppressed. We are currently using super-resolution microscopy to test the hypothesis that SC breakdown progresses from the distal end of the chromosomes to the centromeres, resulting in more dramatic loss of distal pairing and crossing over. Our work has generated a fly model that phenocopies the chromosome-specific meiotic behaviors and defects observed in human female meiosis and could lead to greater understanding of human infertility.

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Initiated, but not completed, double-strand break repair enables segregation of broken chromosomes during mitosis
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Cells must promptly respond to DNA damage to maintain genome stability. DNA breaks occurring in actively cycling cells frequently lead to DNA damage checkpoint-triggered cell cycle arrest prior to the next cell division. However, some DNA breaks do not trigger these checkpoints. More recently, cellular responses to DNA breaks that persist into mitosis have been appreciated, but their molecular regulation remains to be fully determined. Uncovering regulation of these responses is crucial, as failure of such responses can lead to micronuclei, which are aberrant nuclear structures formed from persistent broken DNA that are attributed to disease phenotypes. We have established Drosophila melanogaster rectal papillar cells (hereafter papillar cells) as a highly accessible model to study how broken chromosomes segregate and avoid micronucleus formation during mitosis. We have previously identified that papillar cells lack active interphase checkpoints and therefore frequently enter mitosis with broken chromosomes. Strikingly, these aberrant chromosomes segregate properly despite lacking centromeres/kinetochores. In addition to having inactive interphase checkpoints, we previously showed that papillar cells accumulate persistent DNA breaks during S-phases that occur several days before mitosis. This long delay between DNA breakage and mitosis allows us to easily investigate interphase and mitotic DNA breakage responses separately. Our prior work¹ revealed that conserved Fanconi Anemia proteins underlie micronucleus prevention in dividing papillar cells. Lack of Fanconi Anemia proteins lead to easily detectable tissue development phenotypes, specifically following DNA breakage. Here, we report that initiated, but not completed, double-strand break repair processes enable segregation of broken chromosomes. DNA damage induced during S-phase results in the robust recruitment of DNA damage repair machinery in papillar cells. However, unlike in cells with active interphase checkpoints, which repair double-strand DNA breaks within 24 hours after damage, damaged papillar chromosomes retain specific repair proteins for several days. Further, these repair proteins
show distinct kinetics, either being resolved prior to mitotic onset or persisting into mitosis. These repair proteins are required for segregation of broken papillar cell chromosomes, micronuclei prevention, and cell survival following DNA breakage. We hypothesize that this mechanism is relevant to any cell with inactive cell cycle checkpoints or in cases where DNA damage persists into mitosis. 1. Bretscher and Fox (2016), Dev Cell 37(5):444-57.

P199

**Liquid-liquid phase separation of the Chromosome Passenger Complex triggers auto-activation of Aurora B kinase**

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Many cellular signals are generated by kinases that phosphorylate their own T-loops, which suggests that signaling cascades could be initiated by concentrating a kinase. We are testing the hypothesis that non-membranous organelles generated by liquid-liquid phase separation (LLPS) initiate intracellular signals by triggering such kinase auto-activation. We recently found that the liquid-liquid demixing properties of the Chromosome Passenger Complex (CPC) drives the formation of a non-membranous organelle at inner centromeres and midzones, and here we investigate how phase separation controls Aurora B kinase auto-activation dynamics. We have purified the CPC from *E. coli* and found that in the presence of DNA, chromatin, or RNA, the CPC undergoes spontaneous LLPS at concentrations similar to mitotic cytoplasm. Aurora B kinase auto-activation is slow in soluble CPC, but greatly stimulated by driving the CPC into coacervates. There is a considerable lag phase between CPC coacervate formation and the emergence of kinase activation. However, this lag phase is shortened in the presence of Chk1 kinase, which has been suggested to act upstream of Aurora B signaling. Thus, coacervation acts as a molecular switch to trigger auto-activation in a simple system containing a phosphatase and the CPC. This effect is robust in that it is measurable over a 30-fold range of phosphatase concentrations. Our data demonstrates a mechanism to link the formation of a non-membranous organelle to the generation of an intracellular signal.

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**top-2Is differentially required for the proper maintenance of cohesin localization on meiotic chromosomes in* C. elegans* spermatogenesis and oogenesis**

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During sexual reproduction, haploid gametes are produced from diploid precursors through the specialized cell division of meiosis. Meiosis reduces ploidy by following one round of DNA replication with two rounds of chromosome segregation. In meiosis I, homologous chromosomes segregate, which requires pairing, synapsis, and recombination. In meiosis II, sister chromatids separate from each other similar to mitosis. Type II DNA topoisomerases are enzymes that play a crucial role in chromosome fidelity by disentangling topological problems that arise in double stranded DNA. During mitotic divisions, Topo II enzymes solve topological problems that arise during replication, transcription, sister chromatid segregation, and recombination. However, the exact role Topo II plays during meiosis has not been fully elucidated. Previously, we found that a novel allele of Topo II, *top-2(it7ts)*, disrupts the segregation of homologous chromosomes during the meiotic divisions of spermatogenesis but not
oogenesis. TOP-2 is expressed throughout the germ lines of both spermatogenic and oogenic germ lines, localizing along the lengths of chromosomes during meiotic prophase. In top-2(it7ts) mutants, localization of TOP-2 is disrupted in both spermatogenesis and oogenesis. If TOP-2 localization is disrupted in both germ lines, why is meiotic chromosome segregation aberrant only in spermatogenesis? A major difference between spermatogenesis and oogenesis is the morphology of chromosomes after pachynema. We hypothesize that TOP-2 plays a role in chromosome remodeling/architecture during late meiotic prophase thus facilitating homologous chromosome segregation during spermatogenesis. Analysis of meiotic chromosome architectural components in top-2(it7) spermatogenic germlines, revealed that a meiotic cohesin, REC-8, and the HORMA domain proteins, HTP-1 and HTP-2, are prematurely removed from chromosomes. Additionally, analysis of AIR-2 localization, which is required for REC-8 removal, reveals that it is ectopically localized on chromosomes in top-2(it7ts) spermatogenic germ lines. Normally, AIR-2 localization is mediated through the phosphorylation of histone H3 at threonine 3, which spatially restricts AIR-2 localization to the bivalent short arm. In top-2(it7) spermatogenic germlines, H3 threonine 3 phosphorylation is ectopically localized. These data suggest that TOP-2 is critical for promoting the maintenance of sister chromatid cohesion and accurate chromosome segregation. Currently we are investigating the localization and regulation of additional proteins involved in the sister chromatid cohesion release pathway.

P201

Nhr-23 and spe-44 are essential regulators of meiotic divisions and fb-mo biogenesis during c.elegans spermatogenesis

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In sexually reproducing organisms, such as C. elegans, spermatogenesis is the coordinated process of cellular divisions that gives rise to haploid sperm. Spermatogenesis is regulated by many sperm-specific transcription factors, which are necessary to ensure that the sequence of divisions occurs properly. In this study, we describe the importance of NHR-23, a Nuclear Hormone Receptor that plays a distinct role in upstream regulation of spermatogenesis. NHR-23 was initially identified as a regulator of C. elegans molting but was subsequently found to also be expressed in primary spermatocytes. Using auxin mediated degradation of NHR-23, we found that NHR-23 is required for spermatocyte divisions in both males and hermaphrodites. In the absence of NHR-23, spermatocytes arrest in prometaphase I and do not progress to anaphase I or attempt to partition cellular material and organelles. Interestingly, NHR-23 depleted spermatocytes can form Golgi-derived membranous organelles (MOs) but cannot assemble the major sperm protein (MSP) into fibrous bodies (FBs). To better understand NHR-23’s role in spermatogenesis regulation, we compared the auxin-mediated depletion phenotypes of NHR-23 to that of the previously described spermatogenesis transcription factor, SPE-44. SPE-44 plays a similar but distinct role in spermatogenesis; when depleted, spermatocytes arrest during meiosis and fail to produce MOs or FBs. To further explore FB-MO biogenesis in NHR-23 and SPE-44 depleted male germlines, we examined the localization patterns of the intrinsically disordered protein SPE-18 which functions to assemble MSP into FBs. Preliminary data suggests that SPE-18 is not expressed in SPE-44 depleted germlines, whereas it is expressed but improperly localized in NHR-23 depleted germlines. We
observed similar patterns of expression and improper localization for the MSP accessory protein MSP Fibrous Protein 2 (MFP2); in wildtype worms, MFP2 co-localizes with MSP in the FBs of spermatocytes and in the pseudopods of crawling sperm. These preliminary findings suggest that SPE-44 may directly regulate expression of FB components, while NHR-23 may regulate the expression of assembly factors and post-translational modifiers necessary for FB-MO biogenesis. Co-depletion of NHR-23 and SPE-44 results in minimal expression of MO structures, revealing that these transcription factors have an additive effect on FB-MO biogenesis. Ultimately, this study highlights NHR-23 as a novel spermatogenesis regulator in addition to the previously characterized SPE-44.

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Mer2 binds directly to nucleosomes and axial proteins as the keystone of meiotic recombination
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One of the defining features of sexual reproduction is the recombination events that take place during meiosis I. Recombination is both evolutionarily advantageous, but also mechanistically necessary to form the crossovers that link homologous chromosomes. Meiotic recombination is initiated through the placement of programmed double-strand DNA breaks (DSBs) mediated by the protein Spo11. The timing, number, and physical placement of DSBs are carefully controlled through a variety of protein machinery. Previous work has implicated Mer2(IHO1 in mammals) to be involved in both the placement of breaks, and their timing. In this study we use a combination of protein biochemistry and biophysics to extensively characterise various roles of the Mer2. We gain further insights into the details of Mer2 interaction with the PHD protein Spp1, reveal that Mer2 is a novel nucleosome binder, and suggest how Mer2’s interaction with the axial HORMA domain protein Hop1 (HORMAD1/2 in mammals) is controlled.

P203

Multiple mechanisms lead to chromosome segregation defects in inviable Xenopus hybrids
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Although central to evolution, the cell biological mechanisms underlying the barriers that drive reproductive isolation and speciation are poorly understood. Faulty chromosome segregation that leads to embryonic lethality has been reported in a number of inviable hybrids. This can be due to conflicts between rapidly evolving centromere sequences and the centromere-specific histone CENP-A, which is required for kinetochore assembly and chromosome attachment to the mitotic spindle. Xenopus frog species provide an ideal system to study the basis of this phenomenon, since cross fertilization experiments are easily performed, and mechanisms underlying hybrid incompatibility can be investigated in vitro by combining the sperm chromosomes and egg extracts of different species. We showed previously that hybrids produced when X. laevis or X. borealis eggs are fertilized by X. tropicalis sperm are viable, while the reverse crosses are not. Both inviable hybrids show loss of specific paternal chromosomes. Using the in vitro system, we found that a subset of X. laevis and X. borealis sperm chromosomes lose CENP-A upon passing through a single cell cycle in X. tropicalis egg extract. X. laevis chromosome centromere localization was fully rescued by addition of paternally-matched in vitro translated CENP-A with its chaperone HJURP to the extract. However, although microinjection of these
proteins into embryos reduced mis-segregation and micronuclei, viability was not rescued. Interestingly, *X. borealis* chromosomes showed ultra-fine, stretched DNA morphologies in *X. tropicalis* extracts and CENP-A localization could not be rescued with paternal factors. Ongoing experiments address how inter-species conflicts in transcription and DNA replication contribute to centromere defects and chromosome mis-segregation in inviable *Xenopus* hybrids.

P204

**Traip suppresses chromosome bridges via a mitotic DNA repair mechanism to control brain size**

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Microcephaly is a failure to achieve proper brain size and neuron number during development. Most microcephaly-linked genes function either at the mitotic spindle or in DNA damage repair (DDR). Microcephaly is thought to reflect a loss of proliferation and/or increased cell death. However, few microcephaly genes are well-studied in neurogenesis, and whether the two classes of mutants cause microcephaly via a common pathway is unknown; thus, we are systematically characterizing microcephaly genes in *Drosophila*. Here, we studied the microcephaly gene *Traip*, known to function in DDR, and found that a mitotic DDR function suppresses DNA bridges to control brain size. We show that *Traip* mutant flies have brain structural defects, fewer neurons, and marked loss of neural stem cells (NSCs) via caspase-dependent cell death. *Traip* mutant NSCs have increased DNA damage in interphase, consistent with a DDR function. However, we were surprised to also find polyploid *Traip* mutant NSCs, suggesting mitotic failure. High resolution live fluorescence microscopy of *Traip* mutant NSCs revealed frequent mitotic DNA bridges, providing a possible explanation for the observed polyploid cells via cytokinesis failure. We characterized endogenously tagged *Traip* transgenes; while *Traip* is nuclear in interphase, in mitosis it localizes dynamically on spindles, furrow, and midbody, thus ensuring that it would encounter any DNA bridges. A *Traip* variant lacking the nuclear localization signal (ΔNLS) is evicted from the nucleus and fails to suppress DNA damage during interphase; however, ΔNLS *Traip* localizes properly in mitosis and rescues *Traip* mutant brain phenotypes, showing that a mitotic *Traip* function is sufficient to suppress microcephaly. A possible link between DNA bridges and mitotic failure is the abscission checkpoint; inhibiting the abscission checkpoint suppresses *Traip* mutant brain phenotypes, suggesting this is a key downstream effector. Together, our work challenges current thinking about the relationships between DDR, mitosis and microcephaly by showing that, rather than merely repairing DNA damage during interphase, the primary function of *Traip* is to monitor for and resolve mitotic DNA bridges and thus rescue NSCs from mitotic failure. Now, using *Traip* as a model microcephaly gene, we are using whole brain imaging and 3D analysis to screen for suppressors to uncover more downstream pathways in microcephaly. To date, we have found roles for neuronal stress response, Toll signaling, and caspase-dependent cell death. We are now testing whether these pathways also mediate the phenotypes of other microcephaly genes, including both DDR and mitotic spindle genes, and targeting these pathways as potential therapeutic targets to minimize neuron loss in microcephaly.
P205

**C. elegans maximize the number of euploid progeny in zim-2 mutants with crossover failure on chromosome V.**

**T. Gong, F. McNally; University of California, Davis, Davis, CA.**

Gametes with incorrect chromosome number can lead to embryonic lethality and developmental deficiency if inherited. However, offspring of human trisomy 21 (Down syndrome) mothers are euploid at higher than random frequency while in mouse trisomy models are still lacking. Previous studies have shown that in C. elegans, trisomy IV or X hermaphrodites produce more euploid progenies than expected from random segregation. Here, we study how parents with crossover failure could maximize the number of euploid offspring in C. elegans as crossover failure is likely a more common source of chromosomal abnormality than trisomic gametes. Imaging of a zim-2 mutant revealed that 29.63% of diakinesis oocytes have 6 bivalents and 55.56% have 5 bivalents and two chromosome V univalents. Random segregation should generate 57.41% euploid progeny after mating to a wild-type male. We crossed homozygous zim-2 hermaphrodites that were heterozygous for a chromosome V PCR polymorphism to a wild-type male strain with a third PCR polymorphism on chromosome V. In this experiment, PCR analysis of embryos yields 3 bands for trisomy V, 2 bands for disomy V and one band for monosomy V. Instead of the 57.41% disomic expected from random segregation, we observed 92.59% disomic progeny. This result indicates that C. elegans has the ability to generate mostly euploid progeny from oocytes with a crossover failure on chromosome V. We are currently investigating whether this occurs through correct segregation of achiasmate chromosomes during meiosis or through karyotype correction during embryonic mitosis.

**Cilia and Flagella Structure**

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**TIM, a highly effective CRISPR/Cas9-based targeted insertional mutagenesis method for Chlamydomonas reinhardtii and its utility for characterization of the flagellar protein MOT17**

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Generation and subsequent analysis of mutants is critical to understanding the functions of genes and proteins. Insertional mutagenesis, by which an exogenous DNA is introduced into the cell and then inserts randomly into the genome, disrupting any gene at the point of insertion, has been used widely in the model organism *C. reinhardtii*. However, there is a need for a method to efficiently target and mutate specific genes of interest. Here, we tested strategy and parameters to establish a highly efficient, cost-effective protocol for targeted insertional mutagenesis. The method, termed TIM, utilizes CRISPR/Cas9 to create a DNA break at the targeted site, thus facilitating insertion of exogenous DNA at that site. Our protocol delivers into the cell a Cas9/gRNA ribonucleoprotein (RNP) together with exogenous double-stranded (donor) DNA. The donor DNA contains gene-specific homology arms and an integral antibiotic-resistance gene. When the donor DNA is inserted at the RNP-cut site, it serves both as a selectable marker for mutated cells and as a basis for screening by PCR to identify cell lines in which mutation has
occurred at the targeted site. TIM is flexible with regard to many parameters and can be carried out using either electroporation or the glass-bead method for delivery of the RNP and donor DNA. Using TIM, we were able to generate mutants for six out of six different genes, specifically MOT17, CEP131, and CDPK13, for which no mutants previously have been reported, and FAP70, IFT43, and IFT81. Mutation efficiencies ranged from 40% to 95%; these high efficiencies even allowed us to generate a FAP70 IFT81 double mutant in a single experiment. Characterization of TIM mutants at the DNA level revealed perfect insertions of the donor DNA, as expected for homology directed repair, as well as deletions, duplications, and multiple insertions, all at the intended site. In all mutants examined, the mutations were likely to disrupt the targeted gene. TIM achieves a far higher mutation rate than any previously reported for CRISPR-based methods in *C. reinhardtii*, and promises to be effective for many, if not all, non-essential nuclear genes.

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**Mot17 is a ciliary central apparatus protein associated with the c1a-e-c supercomplex and required for normal ciliary motility**

*Y. Hou, L. Zhao, X. Cheng, N. McNeill, G. B. Witman; University of Massachusetts Medical School, Worcester, MA.*

Cilia are important for cell motility and signal transduction. Motile cilia usually have a microtubule-based 9+2 axoneme consisting of 9 outer doublet microtubules and 2 central microtubules. Attached to the doublet microtubules are large complexes involved in the generation and control of motility, including outer and inner dynein arms, the dynein regulatory complex, and radial spokes. Projecting from the two central microtubules (termed C1 and C2) are other large structures, the functions and compositions of which are less well understood; together, the central microtubules and their projections are called the central apparatus (CA). Cryo-electron tomography of axonemes of the green alga *Chlamydomonas* and the sea urchin *Strongylocentrotus* has revealed an evolutionarily conserved structural complexity to the CA: the C1 microtubule has 6 highly connected projections, termed C1a-C1f, and the C2 microtubule has 5 projections termed C2a-C2e. Currently, 30 *Chlamydomonas* proteins have been confirmed to localize to the CA; over 30 more proteins are predicted to be CA proteins based on proteomic studies. Among them is MOT17, so named because a comparative genomic analysis found that it is restricted to organisms having motile cilia. To learn more about MOT17, we used “TIM,” a CRISPR/Cas9-based method for targeted insertional mutagenesis in *Chlamydomonas*, to disrupt the *MOT17* gene. Cells of *mot17-1* swim slower than wild-type cells, and this swimming defect is fully rescued by MOT17 tagged with HA, indicating that MOT17 has a role in the regulation of ciliary motility. Western blotting confirmed MOT17’s localization to the flagellar axoneme; immunofluorescence microscopy showed that it is located specifically on the CA. When MOT17-HA was immunoprecipitated from a high-salt extract of axonemes, FAP114, FAP227, and calmodulin were co-immunoprecipitated. The latter three proteins are known subunits of a CA supercomplex corresponding to projections C1a, C1e, and C1c, indicating that MOT17 is likely another subunit of this complex. Mass spectrometry analysis of *mot17-1* axonemes revealed that they lacked not only most of MOT17, but also the known C1a-e-c protein FAP101 and two candidate CA proteins, FAP348 and FAP401, indicating that all three are dependent on MOT17 for their assembly into the axoneme, and suggesting that the latter two also are subunits of the complex.
**Evolutionary Origins of Ciliary Axonemal 96 nm Repeat Periodicity**

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All known extant branches of eukaryotes include organisms with motile cilia built on 9 + 2 microtubule scaffolds, in which the motile machinery is arrayed in 96 nm repeats, therefore this repeat architecture was present in the last common eukaryotic ancestor. Our goal is to clarify the evolutionary steps that led to this 96 nm repeat. We propose that the 96 nm period was first generated by superposition of two structural elements, proto-axonemal dyneins that bound with a 24 nm repeat (every 3 tubulin dimers), and radial spokes that bound with a 32 nm repeat (every 4 dimers). This hypothesis is consistent with the 24 nm repeat of outer dynein arms (ODAs), and the 32 nm repeat of radial spokes seen in axonemes simplified through natural selection (Mencarelli et al., 2014), or mutation (Oda et al., 2014). Outer arm dynein extracted by salt will decorate 13 protofilament singlet microtubules at a 24 nm period in the absence of specific microtubule-associated targeting proteins (Haimo, Telser and Rosenbaum, 1979; Mitchell and Warner, 1980) through interaction of an ODA IC subunit with alpha tubulin (King et al., 1991). The ODA docking complex, while needed for high-affinity binding of ODAs to doublets (Owa et al., 2014), is dispensable for in vitro binding of ODAs to doublets with 24 nm periodicity (Oda et al., 2016). Sequence comparisons suggest that proto-axonemal dyneins were homodimers, like cytoplasmic dynein, but evolved by forming heterodimers, followed by differentiation into heterodimeric ODAs and IDAs, and finally, evolution of single-headed IDAs from duplicate copies of one heterodimeric IDA heavy chain gene (Kollmar, 2016). We propose that formation of two rows of axonemal dyneins strongly favored evolution of an ancestral axonemal dynein into two distinct heterodimeric dyneins, both with 24 nm repeat periodicity, each adapted to a different geometry of interaction with the curved surface of an adjacent microtubule track. Later, formation of cylindrical axonemes drove evolution of a central element and radial spokes, with spoke attachment fixed at a 32 nm periodicity. Alignment between dyneins and spokes would then occur only once every 96 nm, providing a platform for evolution of the 96 nm CCDC39/40 ruler proteins, diverse IDAs, and the 96 nm repeat complexes (N-DRC, MIA, CSC) that regulate axonemal dynein activity patterns.

**Analysis of the PKD2-mastigoneme complex in Chlamydomonas reinhardtii flagella**

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Polycyctin-2 (PKD2) is a conserved non-selective cation channel of the transient receptor potential (TRP) channel protein family. In mammals, PKD2 is present in the membrane of cilia in the kidney and embryonic node and mutations result in autosomal dominant polycystic kidney disease and defects in left-right asymmetry. However, the role of PKD2 in cilia remains unclear. Here, we analyzed PKD2 in the flagella of the biphagellate alga *Chlamydomonas reinhardtii*. We previously showed that PKD2 forms two distinct zones in the flagellar membrane: a proximal mobile zone and a distal zone of stationary PKD2. In the latter, PKD2 interacts with mastigonemes, hair-like extracellular polymers of MST1. The PKD2/MST1 complexes are bound to doublet microtubules (DMTs) 4 and 8 of the axoneme and thus are positioned perpendicular to the plane of flagellar beating. Mutants lacking PKD2 or MST1 swim with reduced velocity suggesting a motility related role of the PKD2-mastigoneme complex in the motile flagella of *Chlamydomonas reinhardtii*.
Chlamydomonas (Liu et al. JCB2020). To identify proteins that assist in the formation of the PKD2-MST1 complex, its anchoring to the axoneme and the formation of PKD2 zones, we analyzed the composition of PKD2 complexes in flagella. In detail, we performed mass spectrometric analyses of immunopurified PKD2-mNeonGreen (mNG) complexes isolated from cilia of the pkd2 PKD2-mNG rescue strain and, as a control, wild-type cells (no PKD2-mNG but PKD). Besides PKD2 and MST1, several other flagellar proteins were present in all or most experimental samples. This included mini-PKD2 (GenBank EDO97293), a single transmembrane domain protein of 361 residues with sequence homology to the N-terminal region of PKD2 (1626 residues). A novel mini-pkd2 mutant swims with reduced velocity (like pkd2 and mst1), lacks an MST1 pool near the basal bodies (like pkd2) and has reduced levels of PKD2 in flagella (like mst1). Like the MST1-based mastigonemes, the distribution of mini-PKD2 is limited to Chlamydomonas-like green algae. Taken together, the data suggest that mini-PKD2 is part of the PKD2-MST1 complex in flagella and assists PKD2 in the binding of mastigonemes. We conclude that the analysis of PKD2-mNG complexes immunoprecipitated from isolated flagella allows us to identify novel PKD2 interactors. (supported by NIH grant R01GM110413).

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CODEX: a Neural Network Approach to reveal Signaling Dynamics Landscapes

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Fluorescent biosensors now routinely yield thousands of single-cell, heterogeneous, multi-dimensional cell states trajectories that are difficult to mine for relevant information. We present CODEX (COnvolutional neural networks for Dynamics EXploration), a machine learning based approach to guide data exploration and identify motifs in dynamic cell states in complex time-series datasets. CODEX relies on Convolutional Neural Networks (CNNs) which are trained to classify single-cell trajectories into predefined classes. The classes usually correspond to experimental conditions, or other groups of interest, for which one wishes to reveal signatures in dynamic cell states. Examples of such classes include: drug treatments, mutation profiles and doses in a dose-response challenge. CODEX revolves around the observation that the data-driven features created by the CNNs are shaped around dynamic motifs and trends in the datasets. With the information contained in these features, we obtain an overview of the dynamics relative to each class by the means of different techniques. The final outputs of CODEX comprise: a low-dimensional embedding to visualize the dataset dynamic trends at a glance, a set of representative prototype trajectories for each class, and a collection of discriminative motifs for each class. Despite relying on complex machine learning models, CODEX was designed with simplicity in mind such that it remains applicable to most single-cell time-series datasets. To this end, we propose to use a plain and robust CNN architecture that allows for fast training on consumer-grade hardware, with a moderate amount of data and reduces the required level of machine learning expertise. We show that this method reveals signatures in single-cell dynamics for a range of signaling pathways (ERK, Akt, p53, SMAD) with various classes of interest (nature of dose of input growth, ionizing radiation doses, cell lines). We also demonstrate CODEX flexibility and its wide field of applications on an external dataset which reports the movement speeds of Drosophila.
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**Resource Reports: Using text mining of the biomedical literature to build a database of known antibody issues**

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Antibodies are widely used reagents to test for the expression of proteins in cell biology. However, antibodies are also known sources of reproducibility problems in biomedicine, as specificity and other issues can complicate their use. Information about how antibodies perform for specific applications, including problems with specificity, are scattered across the biomedical literature and multiple websites. We have developed a database through the NIDDK Information Network (dkNET.org) that aggregates information from multiple sources about how antibodies perform. Resource Reports provide a detailed overview of each antibody including published studies, along with any reported issues or validation available. Currently, performance issues and validation information are aggregated from on-line databases like the Antibody Registry, ENCODE, and the Human Protein Atlas. To expand our coverage of reported antibody issues, we recently developed text mining algorithms that can identify specificity issues reported in the literature. We were able to leverage Research Resource Identifiers (RRIDs), unique identifiers for antibodies and other biomedical resources. RRIDs, introduced in 2014 and supported by dkNET, are required by many journals to ensure that authors clearly identify the antibodies within a study. Since RRIDs unambiguously identify the antibodies used, we can match specificity issues with a particular antibody. We developed a deep neural network algorithm and performed a feasibility study on 2223 papers. The results show that our system can accurately perform both nonspecificity classification (weighted F-score over 0.925) and RRID association tasks (weighted F-scores over 0.923). We identified 37 antibodies with 68 nonspecific issue statements. For example, if one were looking for an antibody targeting beta-Amyloid 1-16, from 74 antibodies at dkNET Resource Reports (on 10/2/20), one may select 8 antibodies that had been used (cited with RRIDs) in the literature. With the enhanced alert system, one would find out that "some non-specific bands were detected at ~55 kDa in both WT and APP/PS1 mice with the 6E10 antibody⋯" (RRID:AB_2564652, PMID:30177812). In summary, our results show that it is feasible to expand our Resource Reports alerting service through text mining of published papers.

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**Modeling environmental and genetics effects on cell differentiation kinetics in shmooing yeast**

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Predictive models of cell differentiation kinetics based on environmental and genetic factors are valuable tools for understanding diverse biological systems, from oncology to ecology. Here we describe our effort to build a predictive model of budding yeast pheromone response kinetics. Haploid budding yeasts differentiate from a vegetative growth cell type to a mating competent cell type, called a shmoo, upon environmental exposure to the mating pheromone of the opposite mating type. We hypothesized that the proportion of cells differentiated into a shmoo cell type, as a function of time after pheromone
exposure, would vary across genotypes, temperatures, and pheromone concentrations. For genetic variation, we compared the shmoo dynamics of a lab isolate (S288c) that responds more strongly (at the gene expression level) to high pheromone concentration levels and a clinical isolate (YJM145) that can grow better at higher temperatures. Using a cross design, we systematically measured shmoo dynamics in the two isolates across twelve environments (3 temperatures x 4 pheromone concentrations). Model selection produced the best fit using a multi-parameter non-linear mixed Gompertz function, which is a well-established sigmoid function for fitting growth data. The model included a maximum shmoo proportion parameter (a), a time to reach maximum slope parameter (b), and a slope parameter (c). The mixed model was used to account for the lack of independence caused by repeated measurements from the same yeast culture and to understand the effect of day to day variation. Our model shows that increased temperature decreases the time to reach the maximum slope (parameter b) and increases the slope (parameter c). The model also indicates a trend indifference of the slope (parameter c) between the isolates. Surprisingly, the model revealed no observable effect of varying pheromone concentration, despite previous observations that pheromone concentration affects protein expression for pheromone responsive genes. We expected temperature to positively correlate with an increased mating response rate and there to be a difference between the isolates, however, the lack of correlation for pheromone concentration was unexpected. This could be due to a lack of power from our initial experiments or perhaps highlights a more complex relationship than our model can encompass. Further replication must be done to characterize the exact effects that environmental and genetic factors have on the mating response of yeast.

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Super-long range stable chromosome assembly coordinates gene expression
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A fundamental question in the regulation of mammalian genome expression patterns is how three-dimensional chromosome structures are coupled to dynamic transcriptional activity of specific genes. Our previous computational analyses of RNA-seq and Hi-C data revealed that functionally related genes that were coregulated by common transcription factors tended to be physically close (Zhang et al., PLoS Comp Biol, 15, e1006786 (2019)). Some genes well separated by their genomic distance along a chromosome were nevertheless in physical proximity in the context of three-dimensional chromosome structure. This structural juxtapositioning likely facilitates coordinate epigenetic regulation (Zhang et al. Phys Rev. Lett., 112, 068101 (2014)). To verify the existence of such long-range structures and to examine whether they are transient or stable, we performed live-cell imaging of selected genomic loci labeled with the CRISPR-dCas9 system in human 293 T and A549 cells. We observed a pair of loci separated over 80 Mb to form a stable assembly that fluctuated together during hours of observations. Cells showed heterogeneity in the number of such assembled higher order structures. We hypothesize that these higher order chromosome assemblies are used to coordinate gene regulation. We are experimentally testing this hypothesis.
Critical role of deadenylation in regulating poly(A) rhythms and circadian gene expression

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The mammalian circadian clock is deeply rooted in rhythmic regulation of gene expression. Rhythmic transcriptional control mediated by the circadian transcription factors is thought to be the main driver of mammalian circadian gene expression. However, mounting evidence has demonstrated the importance of rhythmic post-transcriptional controls, and it remains unclear how the transcriptional and post-transcriptional mechanisms collectively control rhythmic gene expression. In mouse liver, hundreds of genes were found to exhibit rhythmicity in poly(A) tail length, and the poly(A) rhythms are strongly correlated with the protein expression rhythms. To understand the role of rhythmic poly(A) regulation in circadian gene expression, we constructed a parsimonious model that depicts rhythmic control imposed upon basic mRNA expression and poly(A) regulation processes, including transcription, deadenylation, polyadenylation, and degradation. The model results reveal the rhythmicity in deadenylation as the strongest contributor to the rhythmicity in poly(A) tail length and the rhythmicity in the abundance of the mRNA subpopulation with long poly(A) tails (a rough proxy for mRNA translatability). In line with this finding, the model further shows that the experimentally observed distinct peak phases in the expression of deadenylases, regardless of other rhythmic controls, can robustly cluster the rhythmic mRNAs by their peak phases in poly(A) tail length and abundance of the long-tailed subpopulation. This provides a potential mechanism to synchronize the phases of target gene expression regulated by the same deadenylases. Our findings highlight the critical role of rhythmic deadenylation in regulating poly(A) rhythms and circadian gene expression.

Dynein: Structure and Function

Long-range Electrostatic Interactions Modulate the Affinity of Dynein for Microtubules

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Dynein is a family of microtubule minus-end directed motor proteins that drives diverse functions in eukaryotic cells, including cell division, intracellular transport, and flagellar beating. Rooted in biophysical theory, processivity, which is a measure of how far a motor protein walks before detaching from its filament, depends on the coupling between the mechanochemical cycles and the duty ratio of a motor protein’s motor domains. Therefore, dynein processivity depends on the interaction between its microtubule-binding domain (MTBD) and the microtubule. Dynein’s MTBD switches between high- and low-binding affinity states as it steps due to structural changes in the MTBD caused by an ATP hydrolysis state-determined registry shift in the coiled-coil stalk domain that connects the MTBD to the motor domain. Recent x-ray crystallography and cryogenic electron microscopy structures suggest that specific salt bridges between the MTBD and the microtubule govern these affinity state shifts. However, our
computational work suggested that non-specific, long-range electrostatic interactions between the MTBD and the microtubule may play a significant role in the processivity of dynein. To investigate this hypothesis, we mutated several negatively charged amino acids remote from the MTBD/microtubule interface to neutral ones and measured the binding affinity using microscale thermophoresis and force-dependent binding kinetics using optical tweezers. We found up to a 10-fold increase in the binding affinity for microtubules in the mutated MTBDs, indicating a potentially critical role for long-range electrostatic interactions in the regulation of dynein. Additionally, we found no directional asymmetry in the unbinding of MTBDs under the influence of external load and a strong dependence on the ionic strength of the solvent. Using mathematical and stochastic modeling techniques, we show that force-dependant unbinding is a combination of slip and catch bonding, depending on the mechanochemical state. Together, our results suggest that long-range electrostatic interactions between the MTBD and microtubules and external forces synergistically regulate the processivity of dynein motors. Furthermore, our results provide insight into the principles underlying the biophysical differences between molecular motors with various physiologically-necessary processivities.

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**Structural basis of cargo binding to dynein and dynactin**

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Dynactin recruits intracellular cargoes to cytoplasmic dynein. The structural basis by which dynein and dynactin are coupled via cargo-associated “activating adaptor” proteins has been determined by high resolution cryo-EM, but the molecular contacts that underlie dynactin-adaptor binding are not completely defined, and how adaptor binding is regulated remains unknown. We and others have reported that dynactin’s p25/p27 (DCTN5/DCTN6) components are part of a cargo-binding module that is essential for early and recycling endosome motility. We have used mutagenesis to investigate how two different p25 structural motifs contribute to the motile and sorting behavior of different endocytic cargoes and in vitro interactions with known coupling adaptors. p25 contains (1) a cluster of lysine residues in a surface loop (K74, K75, K78) that are predicted to interact electrostatically with adaptors, and (2) a conserved C-terminal alpha-helix of unknown function. We explored how these motifs contribute to the movement and sorting of various endocytic cargoes using p25 knockdown/mutant rescue. Transferrin (Tfn)-labeled endosome motility was suppressed by mutations in the surface loop (specifically K78A) and deletion of the C-terminal helix, whereas Tfn recycling/clearance kinetics were only impacted by loss of the C-terminus. p25 depletion also interferes with retromer-dependent trafficking of CI-MPR and spatial segregation from EEA1-positive endosomes. p25 K78 and C-terminal helix are both essential for normal kinetics of perinuclear accumulation, overall rate of efflux and recycling of endocytosed CI-MPR. By contrast, the recycling tubules that return plasma membrane resident proteins (CD98 and CD147) to the surface and help sort these proteins away from Tfn-endosomes required K78, but not the C-terminus. In previous work, we showed that post-Golgi carrier movement requires K74 but not K78 or the C-terminal helix, and that K74 is critical for dynein-dynactin binding to the canonical activating adaptor BICD2. We now extend these findings to explore p25 interactions with the activating adaptor proteins Hook1, Hook2, Hook3, FIP3, girdin and dapple. Once again, we find differential requirements for K74 and K78, and observe that the p25 C-terminus contributes to a variety of interactions. Taken together, our results suggest that p25 interacts with...
different cargo adaptors via a series of distinct contacts that mediate cargo selection and dynein-based movement.

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**Dynein drives post-Golgi apical transport to prevent neuroepithelial progenitor delamination**

**J. Brault**

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In non-polarized cells, post-Golgi transport of RAB6+ vesicles towards the plasma membrane is mediated by Kinesin-1, while the dynein motor is required for their retrograde movement towards the center of the cell. In polarized epithelial cells however, the mechanism for transport from the perinuclear Golgi apparatus to the apical surface remains controversial. This is largely due to the challenge of performing subcellular live imaging in epithelial tissue and to resolve transport of individual vesicles. We here developed a method for fast subcellular live imaging within thick embryonic brain tissue. Neocortical stem cells, known as radial glial cells, are neuroepithelial cells that generate all neurons and most glial cells of the cortex. These cells are highly elongated, enabling the tracking of long-range dynamic events between the basal Golgi apparatus and the apical surface. Using this method, we first demonstrate that microtubules uniformly grow in the apical-to-basal direction, suggesting minus end-dependent transport of post-Golgi vesicles towards the apical surface. Using the pharmacological inhibitor Dynarrestin, as well as overexpression of a p150-glued dominant-negative construct, we show that dynein is critical for apical, but not basal, transport of RAB6+ vesicles. We next tested whether the apical cargo Crumbs, a major regulator of apical domain identity, was transported along this pathway. Using the RUSH system, a synchronized trafficking assay, we show that inhibition of Dynein strongly impairs transport of Crumbs from the Golgi apparatus to the apical surface. We further show that in cortices knocked-out for the dynein partner LIS1, apical localization of the Crumbs complex is severely disrupted. To confirm that these defects are due to altered post-Golgi transport, we generated a double knock-out mouse for RAB6A and B. These mice, which are severely microcephalic, display altered apical localization of the Crumbs complex, phenocopying LIS1 KO. Using live imaging, we show that RAB6 dKO leads to the loss of apical attachment and to delamination of radial glial progenitors. Accordingly, we observe the appearance of basally-located radial glial progenitors that we show continue to proliferate. These cells are highly reminiscent of outer/basal radial glial cells, a hallmark of gyrencephalic species such as primates.

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**Single-particle tracking of cortical dynein reveals mechanisms of force generation and regulation during mitotic entry in the *C. elegans* single-cell embryo**

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During fertilization, the sperm zygote must fuse with the oocyte in order to release its genetic material and form a new embryo. The maternal and paternal pronuclei must then migrate towards each other in
a regulated fashion before they fuse and undergo the first round of cell division. The driving force for this migration is provided through microtubule attachments to the centrosomes. This process is regulated through the joint effort of cytoplasmic contractile forces in the form of actomyosin cortical flows and centrosome pulling forces, where microtubules nucleate from the centrosomes and interact with force generation machinery at the nuclear envelope and at the cortex. This mechanism provides a robust strategy for the cell to accurately position its centrosomes and pronuclei. While it is well known that dynein anchored at the cortex with its force generation machinery plays a central role in the transmission of microtubule forces, direct, high spatiotemporal observation of cortical dynein behaviour is limited, and how cell cycle regulators contribute to cortical force generation in this process is not well understood. Using the C. elegans embryo, we combine high spatiotemporal resolution imaging using Total Internal Reflection Fluorescence (TIRF) microscopy and RNAi depletions with single-particle tracking and a windowed mean squared displacement analysis. With this method, we are able to resolve different populations of dynein behaviour, revealing the mode responsible for force generation. We also identify a mechanistic role for SUR-6, the regulatory subunit of the master mitotic regulator Protein Phosphatase 2A (PP2A-B55/SUR-6) in regulating this process. We conclude that this main counteracting phosphatase of Cyclin-dependent kinase 1 (CDK-1) acts as a regulator of cortical dynein force generation, likely through an interaction with NuMA like protein LIN-5. Taken together, we provide a novel, robust strategy for the classification of cortically tethered, force generating single-particle behaviour in living organisms, and propose a scheme where PP2A regulates dynein by augmenting its ability to bind and unbind to the cortical force generation complex through the dephosphorylation of LIN-5.

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Long-range intramolecular allostery and regulation in the dynein-like AAA protein Mdn1

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Mdn1 is an essential mechanoenzyme that uses the energy from ATP hydrolysis to physically reshape and remodel, and thus mature, the 60S subunit of the ribosome. This massive (>500 kDa) protein has an N-terminal AAA (ATPase associated with various activities) ring, which like dynein has six ATPase sites. The AAA ring is followed by large (>2000aa) linking domains including an ~500aa disordered (D/E-rich) region, and a C-terminal substrate-binding MIDAS domain. Recent models suggest that intramolecular docking of the MIDAS domain onto the AAA ring is required for Mdn1 to transmit force to its ribosomal substrates, but it is not currently understood what role the linking domains play, or why tethering the MIDAS domain to the AAA ring is required for protein function. Here, we use chemical probes, single-particle electron microscopy, and native mass spectrometry to study the AAA and MIDAS domains separately and in combination. We find that Mdn1 lacking the D/E-rich and MIDAS domains retains ATP and chemical probe binding activities. Free MIDAS domain can bind to the AAA ring of this construct in a stereo-specific bimolecular interaction and interestingly this binding reduces ATPase activity. Whereas intramolecular MIDAS docking requires a treatment with a chemical inhibitor or pre-ribosome binding, bimolecular MIDAS docking does not. Hence, tethering the MIDAS domain to the AAA ring serves to prevent, rather than promote, MIDAS docking in the absence of inducing signals.
Endocytosis 1

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Understanding the effect of huntingtin aggregates on clathrin-mediated endocytosis
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Understanding the effect of Huntingtin aggregates on clathrin-mediated endocytosis

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Abstract

Huntington’s disease, an inherited neurodegenerative disorder, is characterized by the formation of neuronal intracellular inclusions. The Huntingtin protein, a 350 kDa protein which is central to this disease, is a product of the HD gene which is ubiquitously expressed, and is conserved across a wide range of species. The underlying cause of this disease is due to an abnormal expansion of the CAG tract (responsible for encoding glutamine, Q) in Exon 1 of the Huntingtin gene, resulting in the formation of intracellular aggregates. Previous studies have shown that Huntingtin aggregates affect endocytosis in yeast and in human HEK 293 cells. However, the underlying mechanism for this remains unknown. In this study we aimed to investigate the effect of wild type and PolyQ repeat-containing Huntingtin protein on clathrin-mediated endocytosis using \textit{Drosophila melanogaster} larval hemocytes as a model system. Our results demonstrate that clathrin-mediated endocytosis is severely affected in the presence of pathogenic aggregates of Huntingtin along with an alteration in the organization of the actin cytoskeleton. This phenotype can be rescued by the overexpression of specific chaperone proteins. Additionally, we also determine that endocytosis in these cells is dependent on a functional actin cytoskeleton. We also describe the effect of other aggregating proteins on endocytosis. Our results reveal a relationship between the endocytic pathway and the actin cytoskeleton in the context of neurodegenerative disorders such as Huntington’s disorder.

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The ubiquitin ligase ITCH functions in clathrin-mediated endocytosis of the EGF receptor

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After activation by specific ligands, epidermal growth factor receptor (EGFR) is endocytosed in clathrin-coated vesicles in a complex with CBL/CIN85 and endophilin, all of which contribute to the internalization of the receptor. ITCH is an E3 ubiquitin ligase bearing a homologous to E6-AP carboxyl terminus (HECT) ligase domain and a proline-rich region (PRR), through which it binds to several SH3 domain-containing proteins. Interestingly, endophilin SH3 binds to ITCH PRR with a relatively high affinity compared to other SH3 domain-containing proteins. ITCH is known to ubiquitinate both CBL and endophilin, providing a potential functional link between the ligase and receptor internalization. While the ubiquitination of CBL causes its degradation in the proteasome, the consequences of endophilin’s ubiquitination are still unclear. In an effort to clarify the role of Itch in EGFR regulation, we generated Itch KO cell lines using CRISPR/CAS9. While blotting for total EGFR, cbl and endophilin showed no
difference in the level of these proteins at rest, there was a slight delay in the degradation of EGFR in ITCH KO cells. Endocytosis assays at different concentrations of fluorescent EGF were performed to compare EGFR internalization in WT and KO cells. In the absence of ITCH, there was a significant decrease in the levels of internalized EGF compared to WT parental HeLa cells. This decrease was most noticeable at lower EGF levels, conditions in which clathrin-mediated endocytosis is recognized as the main entry route of the receptor. Rescue experiments using WT ITCH confirmed the importance of the protein for normal EGF uptake. We generated different ITCH mutants and identified three Arg (R252, 255, 258) critical for endophilin interaction in the PRR. The use of this endophilin-binding defective mutant in rescue experiments allowed us to determine that the ITCH/endophilin interaction is required for normal EGFR endocytosis. Our study describes a new mechanism through which the ubiquitylation machinery regulates EGFR signaling and trafficking.

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Modeling kinetics and control of clathrin recruitment and assembly on membranes
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Clathrin-mediated endocytosis (CME) is an essential pathway used by all eukaryotes for transport across the plasma membrane; it is one of many diverse cellular pathways that require cytosolic proteins to localize and self-assemble on the membrane. A wealth of biochemical, structural, and in vivo imaging data has provided deep insight into the dynamics of endocytosis. Yet because of the complexity of the pathway, it is still remarkably difficult to predict how the stoichiometry of components, membrane bending, or coupling to ATP-driven reactions impacts cargo uptake, and this is where computational modeling can provide important insights. We have developed a structure-resolved model of clathrin recruitment and assembly on membranes that is able to reproduce time-resolved kinetics of in vitro experiments. Our model emphasizes the importance of two sources of cooperativity: 1) adaptor-driven stabilization of clathrin-clathrin interactions and 2) the role of 2D localization in stabilizing all protein-protein and protein-lipid interactions. With this model, we then quantify the dynamics of clathrin nucleation and assembly on membranes, at physiologic concentrations. We specifically show how the ‘stickiness’ of the membrane, as controlled by adaptor protein populations, can sensitively tune the stability and growth of clathrin lattices on membranes. These simulations allow us to predict how changes to the stoichiometry of adaptor proteins and the addition of cargo interactions can control successful clathrin coat growth, or promote disassembly. The theory and models we develop collectively quantify the roles of membrane localization, protein stoichiometry, and cargo capture in controlling transitions from nascent to successful clathrin-coated vesicles.

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Metabolic Control of Clathrin-Mediated Endocytosis by AMPK Impacts Integrin Traffic and Cell Migration
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Clathrin-mediated endocytosis (CME) is the principal mechanism that regulates the diverse set of cell surface proteins, providing broad regulation over key cellular processes such as growth, metabolism, and migration. How CME may be controlled by metabolic cues to regulate the cell surface proteome and downstream signaling remains poorly understood. An important metabolic energy sensor is AMP-activated protein kinase (AMPK), which becomes activated upon metabolic insufficiency to facilitate cellular adaption to metabolic restriction. We previously reported that acute activation of AMPK broadly regulates cell surface membrane traffic, including enhancing the internalization of β1-integrin. We aimed to resolve how AMPK regulates the membrane traffic of β1-integrin to control functions such as cell adhesion and migration. Using total internal reflection fluorescence (TIRF) microscopy, we found that AMPK activation reduces clathrin-coated pit (CCP) initiation rate and size yet increases lifetimes of CCPs and recruitment of β1-integrin therein. Using a FRET-based biosensor, we found that AMPK activation reduces GTP binding by the GTPase Arf6. Using BioID and fluorescence microscopy, we found that the GTPase Arf6 interacts with several clathrin associated proteins, and is recruited to a subset of CCPs. Consistent with regulation of Arf6 GTP binding, AMPK activation triggered a reduction of Arf6 recruitment to CCPs. Using siRNA gene silencing and various assays, we also identified specific GTPase activating proteins (GAPs) that may mediate Arf6 regulation by AMPK to control clathrin endocytosis of β1-integrin. While AMPK activation triggered enhanced clathrin-dependent endocytosis of β1-integrin, it was without effect on clathrin internalization of other cargo receptors. To determine how AMPK activation may specifically regulate β1-integrin membrane traffic, AMPK activation reduced cell migration. Our results identify a novel signaling pathway by which metabolic regulation of CME impacts the function of integrins, impacting cell migration.

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Nutrient dependent regulation of Adapter Protein 2 in clathrin-mediated endocytosis via N-acetylgalactosamine modification
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A key cellular biosynthetic metabolic pathway altered in cancer cells is the hexosamine biosynthetic pathway (HBP) which produces uridine-diphosphate acetylgalactosamine (UDP-GlcNAc) that is subsequently dynamically used as a substrate for the posttranslational modification of ~4000 intracellular proteins with O-linked N-acetylgalactosamine (O-GlcNAc). Protein modification with O-GlcNAc is controlled by a single set of enzymes, O-GlcNAc transferase (OGT) and O-GlcNAc hydrolase (OGA). Clathrin-mediated endocytosis (CME) is the principal mechanism of internalization of membrane proteins from the cell surface. CME initiates by the recruitment of clathrin, the critical adaptor protein AP2, and many other accessory proteins to a small region of the plasma membrane termed a clathrin-
coated pit (CPP). Following recruitment of specific membrane proteins (cargo), some CCPs mature to generate intracellular vesicular carriers, resulting in internalization of cargo. Here, we examine how O-GlcNAc protein modifications may link cellular metabolic status with control of CME and endocytic membrane traffic, and how this novel regulatory axis may be disrupted in cancer cells. Strikingly, we found that the phosphorylation of the critical adapter protein 2 (AP2), an important trigger that controls CCP formation, is regulated by O-GlcNAc modifications and metabolic signals. By using quantitative live-cell imaging of cells expressing fluorescent clathrin and other proteins by total internal reflection fluorescence microscopy, together with experimental perturbations of O-GlcNAc modification and cell metabolism, we measured the impact of O-GlcNAc modification on CCP assembly, efficiency and turnover. We found that enhancing O-GlcNAc protein modification, such as by silencing OGA, resulted in a reduction in CCPs assembly and size. We observed the converse seen when decreasing O-GlcNAc modification by silencing OGT. Consistent with this, we observed that perturbations of OGT and OGA resulted in dramatic remodeling of protein interactions within CCPs, including key proteins clathrin-assembly lymphoid myeloid leukaemia (CALM) and Eps15 interacting protein 1 (EPSIN). Importantly, we also found that activation of PI3K-Akt signaling by the key tumor driver epidermal growth factor receptor (EGFR), known to be dependent on clathrin endocytosis, is also robustly regulated by O-GlcNAc modification. These results suggest that O-GlcNAc modification of proteins required for clathrin endocytosis is a critical metabolic regulator of endocytic membrane traffic.

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The force requirements of endocytic vesicle formation

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Mechanical forces exerted by multiprotein machines are integral to many cellular processes. In clathrin-mediated endocytosis, the major trafficking route for nutrients, signals and membrane components into the cell, mechanical force has to be applied to invaginate the plasma membrane and form an endocytic vesicle. This force is provided by various endocytic proteins, or by oriented polymerization of the actin cytoskeleton when high-energy barrier of membrane deformation has to be overcome. Knowledge of the endocytic force requirements is essential for understanding how the endocytic machinery functions in physiological and pathological conditions. To measure the force needed for endocytosis, we employed FRET (Förster Resonance Energy Transfer) tension sensors, which allow the measurement of pN forces in vivo, and integrated them into the essential force-transmitting endocytic protein Sla2/Hip1R in yeast. We followed force transmitted over Sla2 during individual endocytic events in real time and measured force of ≈10 pN transmitted over Sla2 molecule, hence 450-1300 pN per endocytic event. We then analyzed the role of physical conditions and key membrane-remodeling proteins during endocytic force transmission. We found that endocytic force requirements can be significantly reduced by lowering cell turgor pressure and plasma membrane tension. Enhancement of polymerized actin cytoskeleton also reduced force transmitted over Sla2 protein. Finally, experiments in hypotonic conditions and in cells absent of BAR-domain membrane scaffolds indicated limits of actin force-generating machinery for successful vesicle formation. Altogether, our studies provide critical force values for understanding the mechanobiology of clathrin-mediated and actin-dependent endocytosis, and can be pivotal for understanding mechanics of other membrane remodeling processes carried out by branched actin cytoskeleton throughout the cell.
Establishing and Maintaining Organelle Structure: Multi-Organelle Analysis

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Decoding the variance in integrated intracellular organization of the undifferentiated hiPS cell
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The Allen Institute for Cell Science aims to understand the principles by which human induced pluripotent stem cells (hiPSCs) establish and maintain robust dynamic localization of subcellular structures, and how they transition among distinct states during differentiation in the context of development or disease. One initial step toward this goal is to determine the full range of natural variation in intracellular organization in undifferentiated hiPSCs under normal, unperturbed conditions. To do this we take advantage of ~25 of the endogenous fluorescently tagged hiPSC lines in the Allen Cell Collection (www.allencell.org), each expressing a monoallelic EGFP-tagged protein labeling a particular organelle or structure. We collected thousands of replicate high resolution 3D images of single cells for each structure (over 150,000 in total) and developed image-based assays and segmentation algorithms for quantitative analyses. To measure variation in cell and nuclear shapes, we fit 3D segmented masks using spherical harmonic functions, and then performed a principal component analysis of the spherical harmonic coefficients. We found that the largest overall variation among individual cells in this data set corresponds to how flat (vs. round) cells and nuclei are along the apical-basal axis (Z-direction). This represents variability in how cells pack together within hiPSC colonies, which depends on experimental factors such colony size, plating confluency, and passage number. The second principal shape mode closely corresponds to overall volume, representing cell growth through the cell cycle. We took advantage of the wide range of cell and nuclear sizes and large numbers of structures to perform a survey analysis of intracellular structure size scaling. We found that many key cellular structures scale with cell size, however structures vary in the strength of their scaling relation and whether they scale more strongly with cell size, nuclear size, or the size of other structures. To explore variation in subcellular organization of our EGFP-tagged structures, we parameterized the cytoplasm and nucleoplasm via spherical harmonics to generate spherical maps for each structure in each cell in 3D and then averaged these maps over groups of cells with similar shapes. These maps reveal similarities among subcellular distributions of some subsets of organelles; for example, lysosomes and the Golgi apparatus both occupy a particular region toward the apical surface of the cell, and systematically co-vary in their location across the full range of cell shape variation. This systematic approach has enabled us to determine how subcellular organelle organization varies with changes in cell size and shape in an integrated fashion across ~25 EGFP-tagged subcellular structures.

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In vivo investigation of organelle dynamics in the liver using a novel fluorescent multi-organelle transgenic mouse model
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Organ function relies on fundamental biological processes modulated by external signals and coordinated at the tissue, cellular and subcellular levels. In agreement, defects in organelle activity and impaired inter-organelle communications have been identified as hallmarks of different illnesses including neurodegenerative, cardiovascular and metabolic diseases. The development of advanced fluorescence microscopy tools for in vitro studies has expanded our basic understanding of how organelles sense and respond to extracellular cues (e.g. nutritional signals). However, the intricate regulation of these adaptive responses in the context of the multiscale architecture of organs is poorly understood. This gap in knowledge stems from the lack of readily available tools to simultaneously study multiple organelles in intact tissues. Here, we present a novel fluorescent multi-organelle transgenic mouse model that allows the non-toxic visualization of up to 8 subcellular compartments (mitochondria, peroxisomes, lipid droplets, plasma membrane, endoplasmic reticulum, lysosomes, Golgi, and the nucleus) in anesthetized mice or fixed tissue sections. Using this tool, we investigate how different organelles within the liver parenchyma respond to changes in nutrient availability. We observe that organelle distribution and dynamics are not uniform throughout the liver and follow distinct zonated patterns in normally-fed mice. These patterns dramatically change in response to overnight fasting or nutrient overload. Particularly, nutrient overload after a diet rich in fat and carbohydrates significantly alter the morphology, numbers, and distribution of organelles, some of which are consistent with known hallmarks of liver illnesses such as non-alcoholic fatty liver disease (NAFLD). Overall, the implementation of this novel fluorescent multi-organelle mouse model has allowed, for the first time, the simultaneous visualization of multiple subcellular structures in a living mammal. This tool not only allows the detailed investigation of liver biology at the tissue, cellular, and subcellular level, but also offers a unique opportunity to implement innovative multiplexing approaches to study organelle dynamics in different organs during health and disease states.

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A Pragmatic Approach to Standardizing Ultrastructure Morphology in Tissue and Cell Culture

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Cardiovascular disease (CVD) is the leading cause of mortality in the industrialized and emerging world. Major risk factors for CVD include obesity, dyslipidemia, smoking, diabetes, hypertension, and stroke. Critical mechanisms underlying cardiovascular disease are frequently associated with ultrastructure changes in organelles and can be modeled in vitro and in vivo. Currently, there is no standardized method for quantifying the morphological changes implicated in various pathologies. A growing body of CVD research demonstrates that ultrastructure imaging is vital to understand how genes, proteins, and changes in macromolecule concentrations can alter organelle morphology in vivo and in vitro. Although different microscopy analysis methods have been employed to provide an optimized, in vitro approach to measure organelles and identify dynamic structural changes, a standardized methodology for comparing findings is still lacking. Therefore, we developed a method using transmission electron microscopy (TEM) images that resulted in reproducible calculations to describe organelles’ live morphological changes. We set out to determine an accurate way to quantify mitochondrial hyperbranchning and volume, Mitochondria-Endoplasmic Reticulum Contacts (MERC) length, and percentage coverage, cristae score in comparison to cristae volume density, cristae surface area, and
number. By deleting known mitochondrial dynamic proteins such as OPA-1, MFN-2, and DRP-1, we were able to establish a quantification method that was reproducible and indicative of the expected hallmark mitochondrial phenotypes. Quantification analysis methods were developed using the National Institutes of Health (NIH) ImageJ program, an open-source image processing software designed to analyze multidimensional scientific images, such as TEM and confocal microscopy data sets. Notably, each pixel in the frame is assigned a horizontal and vertical coordinate. Therefore, coordinates of pixels are annotated as pixel 1 = (x₁, y₁) and pixel 2 = (x₂, y₂) and can quantify the dimensions of ultrastructures using geometric calculations. Here, we demonstrate a pragmatic way to measure and analyze fine structural changes in a living cell at higher magnifications using TEM images and ImageJ software.

**Flux, Migration and Compartmentalization**

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**Spatial compartmentalization of HMG-CoA reductases at inter-organelle contacts enhances metabolic flux and the production of sterol-ester rich lipid droplets**

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Eukaryotic metabolism is spatially organized across large length scales to acutely control crosstalk between biochemical pathways, but also to allow for essential substrate sharing between enzymes. While the roles that membrane-bound organelles play in metabolic spatial organization is well characterized, the role of inter-organelle contacts in organizing metabolic reactions remains poorly understood. Here, we show that the yeast nucleus-vacuole junction (NVJ) functions as a platform which spatially regulates mevalonate pathway flux via selective retention of HMG-CoA Reductase (HMGCR) enzymes. HMGCRs enrich at the NVJ in response to acute glucose restriction (AGR), which is dependent on the molecular tethering protein Nvj1. AGR-induced HMGCR partitioning enhances the catalytic efficiency of HMGCR enzymes. During HMGCR NVJ partitioning, mevalonate is preferentially shunted into sterol-ester biosynthesis that generates lipid droplets (LDs) with liquid-crystalline sub-architecture. Loss of Nvj1-dependent HMGCR partitioning can be bypassed by artificially multimerizing HMGCRs, indicating NVJ partitioning enhances HMGCR inter-enzyme associations. We propose a novel mechanism for controlling mevalonate pathway flux via spatial compartmentalization of the rate-limiting enzymes HMGCR, and reveal that AGR induces LDs with remarkable sterol-ester phase-transition properties.

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**Anthrax toxin as a tool to uncover new aspects of mammalian cell biology**

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The study of host-pathogen interactions has uncovered fascinating aspects of mammalian cell biology. This is particularly exemplified by the mechanism through which anthrax toxin enters and hijacks human cells. Dissecting anthrax modus operandi led us to understand the role of post-translational modifications of its receptor, the need for microdomains for toxin-receptor associations, and how the toxin can infect other cells via exosomes. Therefore, we decided to exploit anthrax toxin as a tool to bring to light new aspects of mammalian cell biology. We thus performed two high-throughput
immunofluorescent and siRNA-based screens to improve our understanding on the mechanisms of toxin entry and exosome uptake. Interestingly, the screen for anthrax toxin led us reveal two novel regulation pathways. The first is centered around a protein regulating proprotein convertases, a class of enzymes that cleave anthrax toxin protective antigen. The second pathway relies, instead, on two homologous proteins that control the composition of microdomains. Finally, through our exosome uptake screen, we were able to identify an exosome receptor that can recognize and internalize at least two different types of exosome. All together, these screens and their mechanistic follow-up studies show the extent by which anthrax toxin embodies an ideal model to probe and understand yet-unexplored cell biological pathways.

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**Rafting in a RUSH:membrane microdomains in secretory trafficking**

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**Rafting in a RUSH: membrane microdomains in secretory trafficking**

The determinants of sub-cellular distribution are not known for the vast majority of cellular proteins. Although significant advances have identified a variety of specific motifs responsible for cargo integration into coat/adapter mediated sorting schemes, such motifs are only present on a small subset of membrane proteins. A potential parallel mechanism for organizing membrane protein traffic is sorting by membrane microdomains known as lipid rafts. Such domains are small, dynamic clusters of preferentially interacting lipids and proteins that have been widely implicated in signaling at the plasma membrane (PM) but are likely also present in various endomembranes. Our lab has recently defined the structural determinants of preferential protein partitioning into these ordered membrane domains and shown that raft affinity is required for PM localization of a large subset of membrane proteins. These observations suggested that sorting and trafficking of membrane proteins can be directed by their affinity for a particular membrane environment. To directly assess the role of membrane microdomains in the secretory pathway, we have taken advantage of a robust tool for synchronized protein traffic, known as RUSH (Retention Using Selective Hooks). Here, tagged proteins are retained in specific organelles by a resident “hook”, where they can be quickly released upon introduction of biotin, allowing direct and quantitative analysis of trafficking rates and destinations by fluorescence microscopy. We applied this system to a library of transmembrane domain (TMD) constructs to evaluate the role of raft affinity in secretory traffic, and the machinery involved therein. We find that while TMD-encoded raft affinity is fully sufficient for PM sorting, it is not sufficient for rapid exit from the endoplasmic reticulum (ER), which requires specific cytosolic sorting motifs. However, we find that Golgi exit rates are highly raft-dependent, with raft preferring proteins exiting ~2.5-fold faster than mutants with perturbed raft affinity. We rationalize these observations with a mechanistic, predictive model of trafficking through the secretory pathway, and identify an isoform of the small GTPase Rab6 as a central regulator for the Golgi-PM trafficking of raft proteins. These observations highlight a central role for lipid rafts in sorting in the secretory pathway and establish the core machinery for raft-mediated cellular trafficking from Golgi. The proposed model helps to understand how TMD proteins migrate from ER to their final post-Golgi destination.
Defining the role of perilipins during metabolic transitions in the hepatic lobule
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Nonalcoholic fatty liver disease (NAFLD) is an expanding health crisis affecting one-third of adults in developed countries and is the fastest rising cause of hepatocellular carcinoma (HCC). The pathogenesis, however, remains poorly understood and therapy is limited. The vast metabolic activities the liver fulfills are spatially divided in the hepatic lobule; particularly, β-oxidation and fatty acid (FA) synthesis occur in periportal (PP) and pericentral (PC) hepatocytes, respectively. The metabolic compartmentalization allows efficient metabolic transition between FA de novo synthesis and FA β-oxidation during periods of feeding and fasting. However, metabolic compartmentalization is disrupted in liver disease and the liver cannot undergo sufficient metabolic transitions. FAs are stored in lipid droplets (LDs) that serve as key regulatory hubs for FA storage and utilization via their association with different proteins and organelles. The perilipin (PLIN) family consists of five conserved members that associate with LDs and participate in their biogenesis, stabilization and utilization. Overexpression of PLINs have been associated NAFLD and HCC. However, their role in metabolic transitions in the liver is not known. Here we investigated the distribution of PLINs in the hepatic lobule under fed/fasted conditions. We hypothesized that the distribution of different PLIN proteins will differ in a manner reflecting their specific activities within PP or PC cells. Using fluorescence-activated cell sorting (FACS), we isolated PP and PC hepatocytes and evaluated the expression of PLINs. We found that PLIN5 and PLIN4 were enriched in PP and PC cells, respectively in normally fed mice. Consistently, immunofluorescence studies confirmed that PLIN5 is highly expressed in PP cells while LDs were more commonly observed in PC cells. In overnight fasted mice (12-hour), large LDs were evident together with PLIN5 throughout the hepatic lobule. Interestingly, PLIN1 and PLIN2 were primarily expressed in PC hepatocytes. Taken together, our findings suggest that 1. PLIN distribution and association with LDs changes during metabolic transitions. Ongoing studies aim to deepen our understanding of the role of PLINs during metabolic transitions in PP and PC hepatocytes by performing proteomic and post translational modification analyses in fed and fasted mice. Thereby, understanding the role PLINs fulfill in regulating FA utilization and storage will yield novel insights into the mechanism(s) of liver dysfunction in NAFLD.

Nir1 is a Novel ER-PM Junction Tether and a Positive Regulator of PM PI(4,5)P2
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Endoplasmic reticulum-plasma membrane (ER-PM) junctions are subcellular loci characterized by the close and stable apposition of the ER to the PM. ER-PM junctions have been demonstrated to facilitate Ca^2+ signaling/homeostasis as well as lipid signaling and metabolism. However, our knowledge regarding the molecular factors required to maintain and modulate ER-PM junctions is limited. Here, we describe a previously unidentified tether that is required for ER-PM junction maintenance as well as supports non vesicular-mediated PI(4,5)P_2 homeostasis at the PM. While we validated that membrane targeting domains are functionally conserved in Nir proteins, Nir1 is distinct in that it is enriched at ER-PM junctions in resting cells. Manipulation of Nir1 levels in the cell via overexpression or transient knock-
down drives remodeling of ER-PM junction properties such as size and density. Additionally, Nir1 lacks a phosphatidylinositol transfer protein (PITP) domain, yet Nir1-deficient cells exhibit reduced P(4,5)P₂ replenishment at the PM. Further, Nir1 expression potentiates targeting of the lipid transfer protein (LTP) Nir2 to ER-PM junctions during receptor-mediated signaling. Lastly, live-cell and co-immunoprecipitation data identify Nir1 as an interactor of Nir2. Results from this study reveal Nir1’s role as a novel ER-PM junction tether as well as a positive regulator of PM P(4,5)P₂ and the LTP Nir2. These findings provide greater mechanistic insight into non-vesicular mediated lipid transfer at ER-PM junctions.

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Last step in the path of LDL cholesterol from lysosome to plasma membrane to ER is governed by phosphatidylserine

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Animal cells acquire cholesterol from receptor-mediated uptake of low-density lipoprotein (LDL), which releases cholesterol in lysosomes. The cholesterol moves to the endoplasmic reticulum (ER), where it inhibits production of LDL receptors, completing a feedback loop. Here we performed a CRISPR-Cas9 screen in human SV589 cells for genes required for LDL-derived cholesterol to reach the ER. We identified the gene encoding PTDSS1, an enzyme that synthesizes phosphatidylserine (PS), a phospholipid constituent of the inner layer of the plasma membrane (PM). In PTDSS1-deficient cells where PS is low, LDL cholesterol leaves lysosomes but fails to reach the ER, instead accumulating in the PM. The addition of PS restores cholesterol transport to the ER. We conclude that LDL cholesterol normally moves from lysosomes to the PM. When the PM cholesterol exceeds a threshold, excess cholesterol moves to the ER in a process requiring PS. In the ER, excess cholesterol acts to reduce cholesterol uptake, preventing toxic cholesterol accumulation. These studies reveal that one lipid—PS—controls the movement of another lipid—cholesterol—between cell membranes. We relate these findings to recent evidence indicating that PM-to-ER cholesterol transport is mediated by GRAMD1/Aster proteins that bind PS and cholesterol.

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Miro recruits VPS13D to mitochondria

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Mitochondria, which are excluded from the secretory pathway, depend on lipid transport proteins for their lipid supply from the ER, where most lipids are synthesized. In yeast, the outer mitochondrial membrane GTPase Gem1 is an accessory factor of ERMES, an ER-mitochondria tethering complex that contains lipid transport domains and plays a function, partially redundant with VPS13, in lipid transfer between the two organelles. In metazoa, where VPS13, but not ERMES, is present, the Gem1 orthologue Miro has been linked to mitochondrial motility but not to lipid transport. Here we show that Miro
recruits to mitochondria the lipid transport protein VPS13D which, like Miro, is an essential protein in mammals, and whose localization had remained elusive. We also show that VPS13D can tether mitochondria to the ER in a Miro- and VAP-dependent way. These findings reveal a so far missing link between function(s) of Gem1/Miro in yeast and higher eukaryotes, where Miro is a Parkin substrate, with potential implications for Parkinson’s disease pathogenesis.

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Transbilayer coupling and membrane curvature changes underlie sorting of lipids and proteins into the HIV virus membrane

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Particles that bud off from cell surface, including enveloped viruses and microvesicles, typically have a unique protein composition distinct from that of the originating host cell plasma membrane (PM). This selective protein composition is particularly important for viruses such as HIV, since it enables the virus to evade host immune response and infect other cells. But how proteins sort into budding HIV remains unclear. We used a combination of real-time imaging of single virus assembly and STED based fluorescence correlation spectroscopy (STED-FCS) to dissect the mechanisms of protein sorting into HIV membranes. An existing view is that protein incorporation into HIV membrane is a passive process, reflecting the pre-existing protein distribution at viral assembly site. We discovered that, contrary to this view, multimerizing HIV Gag, the viral structural protein, actively remolds the viral assembly site membrane, generating a phase-separated ordered lipid domain. Specific proteins localize into the assembly site domain via a lipid-based partitioning mechanism. In studying this process, we found that assembly site phase separation is driven by the combination of a transbilayer coupling process and increasing membrane curvature. STED-FCS experiments showed that HIV Gag’s interactions with PIP2 at PM inner-leaflet led to clustering of PIP2 at the assembly site. We provide evidence that the ordering of inner-leaflet lipids induced by Gag is transmitted to outer-leaflet by transbilayer interactions between the acyl chains of PIP2 and long, saturated acyl chains of outer-leaflet molecules such as GPI-anchored proteins and sphingolipids. This appears to register the lipid-ordering in the two leaflets, decreasing the entropy of mixing and driving the formation of an ordered lipid domain that spans both leaflets. Use of curvature-deficient Gag mutants revealed that curving of the assembly site by the Gag lattice is also necessary for progressive ordering of the assembly site and protein sorting. The increased curvature of assembly site stimulated shape-based sorting of lipids and proteins, and appeared to stabilize the ordered assembly site domain by reducing line-tension at its boundary with bulk PM. Together, these data support a physical mechanism based on transbilayer-coupling and membrane curvature for selective incorporation of proteins and lipids into the HIV viral membrane. A similar mechanism driven by multimerizing structural proteins and membrane curvature may be involved in budding of other enveloped viruses and microvesicles, and may facilitate various intracellular budding processes associated with membrane trafficking.
Gene Expression Machinery in Development

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**Nascent Wars: Opposing effects of RARE enhancers on coordinate HoxB transcription**

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Normal body patterning depends upon the proper expression of Hox genes during development. In mammals there 39 Hox genes divided into 4 clusters. These genes must be tightly regulated so that appropriate levels and spatio-temporal expression profiles are established to facilitate their input into downstream effectors and pathways they modulate. This occurs in part due to enhancers acting both locally and long-range within and around the Hox clusters. Additional transcriptional complexity arises due to the presence of long non-coding RNAs (lncRNAs) that are embedded within and around the Hox coding regions. Within the HoxB cluster, we have identified several enhancers that contain retinoic acid response elements (RAREs) and demonstrated that they are involved in coordinate/global regulation of multiple coding genes and lncRNAs in the cluster. A question that arises is how do these shared enhancers influence the dynamic transcriptional events that modulate de novo activation of multiple genes? To address this, we optimized the use of a single molecule florescent in situ hybridization technique (smFISH) in mouse embryo sections to monitor ongoing or nascent transcripts of coding and non-coding RNAs within the HoxB cluster. In order to quantify imaging data in an unbiased and high throughput manner, we applied a deep learning approach. This analysis was performed in neural tissues of both wildtype and RARE mutant embryos to assess the impact of these enhancers on patterns of transcription. We do not observe simultaneous nascent transcription of all HoxB genes in individual cells, as diverse combinations of active signals are only observed in subsets of cells. This is consistent with rapid dynamics in transcriptional events. Mutations in RARE enhancers alter the balance of transcriptional events. For example, in DE-RARE mutants, the number of nascents for the anterior HoxB1 gene increases while the posterior HoxB9 transcripts are significantly reduced. In contrast, B4U-RARE mutants, we observe that all transcripts are significantly increased, suggesting this enhancer could potentially be repressing transcripts in the tail segment. Intriguingly, in double DE-B4U RARE mutants, the levels of nascent transcripts of HoxB coding genes are restored similar to wild-type levels. This suggests these two RARE enhancers may exert antagonistic inputs on each other’s activity, and in the double mutant their effect is neutralized. Our results show that while each RARE enhancer has specialized inputs into regulating nascent transcription of multiple HoxB genes, they also interact to modulate the balance of coordinate transcription in order to fine tune the expression of HoxB genes during neural development.

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**Crip2 a novel Cu-binding LIM domain protein that contributes to skeletal muscle differentiation**

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Cu is an essential cofactor for cellular processes, such as cellular respiration and oxidative stress regulation. The imbalance of Cu homeostasis promotes the neurological deficiencies and defects in
skeletal muscle development and functions, like Wilson’s and Menkes diseases. Evidence points to that those muscular deficiencies can result from impaired systemic metal transport. However, it is not clear whether these myopathies are instead the result of dysfunctional Cu-dependent transcriptional mechanisms. In eukaryotic cells, the evidence for Cu and Cu-transcription factors (Cu-TFs) is limited to metal homeostasis, and only three Cu-TFs have been identified in mammalian cells. Using an unbiased metallocproteomic approach of native gel electrophoresis coupled to mass spectroscopy in differentiating primary myoblasts, we identified a novel protein with the potential to bind Cu, CRIP2. This CRIP2 protein is annotated as a Zn-binding LIM domain protein that regulates NF-κB-mediated proangiogenic cytokine transcription. However, no information is available on the biological role of CRIP2 in the skeletal muscle lineage. Sequence analyses showed that CRIP2 might bind Cu at various metal-binding sites, which were confirmed by copper-binding determinations using atomic absorbance spectroscopy and in vitro metal binding affinity assays. In proliferating and differentiating myoblasts, Cu treatment increases CRIP2 expression, suggesting a role for CRIP2 in myogenesis that may be associated with Cu availability. CRISPR/Cas9 deletion of CRIP2 impaired differentiation with no evident effect in myoblast proliferation. The data supports the hypothesis that proteins that are annotated as Zn-binding proteins may bind Cu with higher affinity with essential implications in developmental processes.

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Mammalian SWI/SNF chromatin remodeler is essential for reductional meiosis in males
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Meiosis is critical to the formation of haploid gametes and therefore sexual reproduction. The role of chromatin in this process is evidenced by the growing list of chromatin regulators that impact meiosis. This includes the mammalian SWI/SNF (SWItch/Sucrose Non-Fermenting) ATP dependent chromatin remodeling complex, which is required for mouse spermatogenesis. In previous studies we have shown that BRG1, a SWI/SNF catalytic subunit, promotes progression through meiotic prophase-I, by regulating germ line transcription. Interestingly, SWI/SNF is composed of multiple subunits (~10-14), some of which identify biochemically distinct subcomplexes, whose functions in meiosis remain unknown. Here, we identify a role for the PBAF (Polybromo - Brg1 Associated Factor) complex in the regulation of meiotic cell division. The germ cell-specific depletion of PBAF DNA binding subunit, ARID2 resulted in a metaphase-I arrest. Arid2KO metaphase-I spermatocytes displayed defects in chromosome organization and spindle assembly. Additionally, mutant centromeres were devoid of Polo-like kinase1 (PLK1), a known regulator of the spindle assembly checkpoint (SAC). The loss of PLK1 coincided with a failure to target cell division associated chromatim modifications such as Histone H3 threonine3 phosphorylation (H3T3P) and Histone H2A threonine120 phosphorylation (H2AT120P) to centromeric regions. Consistent with the known role of these histone modifications in chromosome passenger complex (CPC) recruitment, Arid2KO metaphase-I chromosomes display defects in CPC association. We propose that ARID2 facilitates metaphase-I exit by regulating spindle assembly and centromeric chromatin.
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Chromosome Segregation Fidelity is Inherently Low in Pluripotent Human Embryonic Stem Cells Compared to Somatic Cells
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During cell division chromosomes must be accurately segregated to produce daughter cells with the correct numbers of chromosomes whereas segregation errors generate aneuploid cells with abnormal numbers of chromosomes. In normal human somatic cells, chromosome segregation errors and aneuploidy are rare. In contrast, in human totipotent and pluripotent embryonic cells meiotic and mitotic errors are common, resulting in aneuploidy being the leading cause of miscarriages and birth defects. Yet, we do not understand the mechanisms responsible for this, particularly for mitotic errors. Here, we directly compare cell division between pluripotent human embryonic stem cells (hESCs) and somatic cells to investigate the mechanisms that cause chromosome mis-segregation in hESCs. Using quantitative live-cell imaging and chemical approaches, we show that mitotic errors, including lagging chromosomes, are significantly elevated in hESCs compared to somatic cells. Moreover, we show that increasing mitotic duration or decreasing chromosome microtubule attachment stability in hESCs decreases the frequency of mitotic errors. These results demonstrate that hESCs do not efficiently correct chromosome microtubule attachment errors and that multiple pathways contribute to the inefficient error correction. Collectively, our data demonstrates that pluripotent embryonic cells are inherently different from somatic cells with respect to mechanisms that support chromosome segregation fidelity. Importantly, these results offer new strategies to improve the genome stability of human embryonic cells grown in culture that is critical to the success of reproductive and regenerative medicine therapies.

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Transcriptional regulation of miR-1 and its regulatory role in development
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MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression by binding to the 3’UTR and silencing translation. miRNAs have critical regulatory roles in embryogenesis and the physiological functions of cells. Dysregulation of miRNA levels has been linked to cancer, cardiovascular and neurodegenerative diseases. While some progress has been made in understanding the function of some miRNAs, little is known about their transcriptional regulation. This study focuses on an evolutionarily conserved miRNA, miR-1. miR-1 is a known regulator of cardiac development and function, regulation of skeletal muscle proliferation and differentiation and blood vessel formation by suppressing VegfA. Inhibition of miR-1 resulted in defects in development and function of skeletogenic primary mesenchyme cells (PMCs) in sea urchin embryos. miR-1 is predicted to have binding sites within the 3’UTR of Vegf3 and skeletogenic transcription factors Tbr and Ets1. Interestingly, Tbr knockdown resulted in a decrease of miR-1 levels while inhibition of Vegf signaling with axitinib increased miR-1. This potentially suggests that miR-1 suppresses key skeletogenic transcription factors and signaling, and
it itself is transcriptionally regulated by components of the skeletogenic PMC gene regulatory network. Identifying miR-1 function and transcriptional regulators will provide a better understanding of how transcriptional and post-transcriptional regulatory mechanisms cross-regulate to make a functional embryo. This work will serve as a paradigm to understand miRNA function and its regulation in other organisms.

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**Conserved transcriptional logics of Nr2f genes in atrial development**

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Nr2f transcription factors show conserved expression in atrial cardiomyocytes (ACs) and centrality to regulation of vertebrate atrial development. Nevertheless, the regulatory logic underlying *Nr2f1/2* gene expression in vertebrate ACs remains obscure. Results: To understand the transcriptional regulation of *Nr2f1/2* genes in ACs, we first cloned an ~1.8 kb region of the zebrafish *nr2f1a* putative promoter and 5’UTR that is highly conserved among loci of gnathostome *Nr2f1* and *Nr2f2*, which we identified as whole-genome duplicates. However, this region showed brain and pan-cardiac expression, suggesting other conserved cis-regulatory modules (CRMs) must direct AC-specific expression. Then, we used Assay for Transposase Accessible Chromatin with high-throughput sequencing (ATAC-seq) to compare open chromatin for the genomic locus of zebrafish *nr2f1a* (730 kb) in isolated ACs to vertebrate *Nr2f1* and -2 loci using VISTA and manual alignments. This approach identified a 280 bp enhancer 3’ to *nr2f1a*, which we call 3’reg1, conserved in gnathostome *Nr2f1* orthologs and able to direct GFP expression in ACs of stable transgenic embryos. Integrating transcription factor binding site analysis and manual alignments identified conserved putative sites within 3’reg1 for FoxF, Gli and Tcf factors. Three FoxF deletions (ΔFoxF) and ΔGli reduced 3’reg1 expression in ACs. Otherwise, ΔTcf-1 expanded 3’reg1 in all the heart and ΔTcf-2 reduced its expression in ACs. Intriguingly, drug treatments proved the 3’reg1 responsiveness to Hh and Wnt pathways. Interestingly, human 3’reg1 region drove expression in zebrafish ACs. Conclusion: We identified the gnathostomes’ 3’reg1-nr2f1a atrial enhancer, which is controlled by Hh and Wnt pathways through one Gli and two Tcf sites, respectively, with the possible implication of FoxF transcription factors. Our data pave the way to understand the gene regulatory networks driving vertebrate atrial development and the etiology of human congenital heart defects.

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**The role of CREB3L1 in bone development**

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The bZIP transcription factor cAMP-responsive element binding protein 3-like-1 (CREB3L1) is essential for healthy bone formation. Patients with CREB3L1 mutations exhibit osteogenesis imperfecta, while CREB3L1⁻/⁻ mice exhibit defects in bone density and fracture healing. However, the transcriptional repertoire of CREB3L1 is poorly characterized. Unlike most transcription factors, CREB3L1 is localized to the endoplasmic reticulum (ER), where it is transcriptionally inert. During endoplasmic reticulum stress signaling, CREB3L1 is activated by transport to the Golgi, where it undergoes regulated intramembrane
proteolysis (RIP) to release the N-terminal transcriptionally active fragment (TA). The TA fragment is then translocated to the nucleus, where it regulates transcription of target genes. To identify the transcriptional targets of CREB3L1, we have generated two zebrafish (Danio rerio) mutants via CRISPR/Cas9-mediated truncations, which allow for endogenous expression of modified CREB3L1. The first truncation is in exon 2 (CREB3L1^{−/−}) and encodes amino acids 1-63 of CREB3L1. Any translated product of this allele lacks the bZIP domain, and is therefore non-functional. The second truncation is in exon 9 (CREB3L1^{TA/TA}), and the translated product encodes amino acids 1-374 of CREB3L1 and contains the entire transcriptionally active region. To examine the effects of CREB3L1 deletion or expression of the transcriptionally active TA fragment on bone development, we compared wild-type, CREB3L1^{−/−} and CREB3L1^{TA/TA} alleles by μCT and histology of adult fish. We also utilized Alizarin red and Alcian Blue whole-embryo staining. In validation of our model system, CREB3L1^{−/−} fish had less dense bones than wild-type animals, while the CREB3L1^{TA/TA} fish had denser bones. Using this model, we will identify the transcriptional repertoire of CREB3L1 through RNASeq analysis of whole embryos, as well as osteoblasts isolated from larvae expressing the three alleles. In addition, we will utilize CHIPSeq and BioID approaches in the mouse MC3T3 osteoblastic cell line model of bone development. These cells, which have been modified to express the human TA fragment of CREB3L1, will be used to identify CREB3L1 binding sites on DNA and putative transcriptional co-factors. In combination, these methods will work towards defining the transcriptional niche of CREB3L1, as well as its role in bone development.

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**Hox genes function post-embryonically to coordinate size-dependent adult tissue segmentation with asexual reproductive behavior**

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Hox genes are a highly conserved transcription factors renowned for their roles in segmental patterning of the embryonic anterior-posterior (A/P) axis. Emerging evidence for Hox gene expression and function in postnatally derived structures has fueled interest in their additional roles beyond embryogenesis. We recently characterized how size-dependent postnatal patterning underlies asexual reproduction in the planaria, Schmidtea mediterranea. These highly regenerative flatworms undergo transverse fission - an asexual reproductive behavior in which posterior tissue fragments are sequentially torn off in order to regenerate clonal progeny. Size-dependent patterning segments the adult body antero-posteriorly to allocate tissue for fission progeny while remodeling the brain to modulate fission behavior. The molecular mechanism integrating animal size with the establishment and coupling of these postnatal structures is still unclear. We hypothesized that Hox genes mediate A/P directed tissue segmentation and transverse fission behavior underlying asexual reproduction. RNAi of each of the 13 planarian Hox family members in adult animals revealed 5 Hox genes (hox1, hox3a, hox3b, lox5b and post2b) required for asexual reproduction. Amongst these, RNAi of hox3 genes resulted in supernumerary segments, while RNAi of post2b eliminated segmentation altogether. The opposing roles of hox3 and post2b in segmentation were paralleled in the regulation of fission behavior. RNAi of hox3 increased the frequency of fission behavior initiation, while RNAi of post2b eliminated fission behavior entirely. To determine the downstream effector genes mediating Hox gene function, we performed RNAseq analysis of hox RNAi animals to identify differentially expressed genes and assess their functions in asexual reproduction. We identified 24 Hox regulated effector genes required for asexual reproduction. Notably,
the majority of identified genes were conserved from planarians to humans and had putative functions in the nervous system. We next used scRNAseq expression analysis to resolve cell populations in which *Hox* genes were co-expressed with their cognate functional effectors. This analysis identified a set of radially layered parenchymal cell populations which may underlie the integration of animal size with asexual reproduction. In conclusion, our study identifies post-embryonic roles for planarian *Hox* genes as couplers of size-dependent adult tissue segmentation and behavior in addition to providing the first evidence that *Hox* genes function to regulate asexual reproduction. Furthermore, our findings highlight how study of planarian asexual reproduction represents an experimentally tractable means of resolving conserved mechanisms of action for *Hox* genes.

**P246**

**Tbx1 suppresses transcriptional activity of RUNX2 and regulates chondrocyte maturation**

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The synchondrosis in the cranial base is an important growth center for the craniofacial region. The sphenoid-occipital synchondrosis (SOS) contributes to the embryonic and postnatal elongation of the cranial base, until its ossification between the ages of 16 and 18 years in humans. Abnormalities in the SOS affect the development of adjacent regions, including the craniofacial skeleton. Here, we report that the transcription factor TBX1, the candidate gene for DiGeorge syndrome, is expressed in mesoderm-derived chondrocytes and plays an essential and specific role in SOS development by inhibiting the expression of genes involved in chondrocyte hypertrophy and osteogenesis. In *Tbx1*-deficient mice, SOS was completely mineralized at birth. TBX1 interacts with RUNX2 and suppresses its transcriptional activity. Indeed, deleting *Tbx1* triggers accelerated mineralization due to accelerated chondrocyte differentiation, which is associated with ectopic expression of downstream targets of RUNX2 in the SOS. These findings reveal that TBX1 acts as a regulator of chondrocyte maturation and osteogenesis during the SOS development. Thus, the tight regulation of endochondral ossification by TBX1 is crucial for the normal progression of chondrocyte differentiation in SOS. This work was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI [20K09901 to N.F.]; and Astellas Foundation for Research on Metabolic Disorders [to N.F.]. **Keywords:** 22q11.2 Deletion Syndrome, Skull Base, Cartilage, Mesoderm. **References** (1) Funato N, Srivastava D, Shibata S, Yanagisawa H. TBX1 regulates chondrocyte maturation in the sphenoid-occipital synchondrosis. *J. Dent. Res.* 2020; 99(10): 1182-1191. (2) Funato N. New insights into cranial synchondrosis development: a mini review. *Front. Cell Dev. Biol.* 2020; 8(706): 1-9.

**Higher-Order Actin-Based Structures**

**P247**

**Cdc42ep3 crosslinks septin and actin filaments**

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The septins are a group of filament-forming and GTP-binding proteins. Dynamic assembly and disassembly of septin filaments are implicated in cytokinesis and in the formation of contractile actin-
myosin structures called stress-fibers. Within these structures, septin-actin interactions can be direct or mediated by adaptor proteins. One proposed septin-actin adaptor protein is Cdc42EP3, which is upregulated during the conversion of fibroblasts into cancer associated fibroblasts and promotes the assembly of stress-fibers and septin filaments. Cdc42EP3/BORG2 is one of the five human Cdc42 Effector Protein (Cdc42EP) family members, which are also known as the Binder of Rho GTPase (BORG) proteins. While the cell biology is consistent with Cdc42EP3 serving as an adaptor protein, the biochemical details of the interaction with actin are poorly understood. Here, we tested this inter-filament adaptor protein hypothesis through in vitro reconstitution. Using a combination of hydrodynamic analysis and spectroscopic probes, we found that Cdc42EP3 binds to septins and actin, both as pairwise interactions and simultaneously. By imaging the resulting filament networks, we found that Cdc42EP3 condenses actin filaments tightly onto septin filament networks. In addition, Cdc42 binding modifies the Cdc42EP3 affinities for both septin and actin. Thus, our data establish Cdc42EP3 as an adaptor protein that is capable of bridging between distinct cytoskeletal networks.

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Nonmuscle Myosin II Drives Actomyosin Bundling at Nascent Adherens Junctions

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Actomyosin-based structures assembled at apical E-cadherin (ECad)-mediated zonula adherens (ZA) modulate border tension to control cell shape during morphogenesis and homeostasis. The molecular mechanisms underlying assembly of the actomyosin structures at the ZA remain unclear. To investigate this, we used ZO-KD MDCK cells as a model, since they form robust and highly organized actomyosin structures at the ZA, with tightly bundled actin filaments running along bicellular borders and a sacromeric array of myosin generating contractility. We triggered junction formation via calcium-switch, and applied super-resolution microscopy to study dynamic re-assembly of the junction-associated cytoskeleton in 3-D. Upon removal of calcium, ECad is internalized, and cell junctions and the associated actomyosin array disassembled. Re-addition of calcium returned Ecad to new cell contacts, membranes gradually zipped apically, and cell junctions and associated actomyosin arrays reformed. We were surprised to find that myosin formed highly organized arrays early in the process, when the actin cytoskeleton is still much less well-organized. At young junctions, myosin mini-filaments assembled into extensive stacks along the cell cortex. These were apical to the re-assembling junctions, and the stacks can be microns in XY dimension while remaining in ~0.5 micron in the Z dimension. As junctions matured, the length of the myosin stacks shortened in XY, and actin filaments assembled into the bundled apical arrays seen before perturbation. We used pharmacological or genetic approaches to test two mechanisms potentially regulating formation of the organized ZA actomyosin array: 1) that it is driven by new actin polymerization, or 2) by reorganization/bundling of actin filaments by myosin. Surprisingly, the actin nucleators Arp2/3 and Formin and the actin elongator Ena/VASP all appeared dispensable for this process. The Arp2/3 results were especially intriguing, since previous studies suggested Arp2/3 maintains actin at the ZA during junctional homeostasis. However, Arp2/3 inhibition did disrupt an actin pool associated with more basolateral Ecad. In contrast, when we inhibited myosin phosphorylation or its ATPase activity, formation of the ZA actomyosin array was disturbed. Inhibiting ROCK, but not MLCK, prevented assembly of bundled F-actin. When myosin ATPase activity was
inhibited, sarcomeric myosin structures failed to form but Ecad-mediated adhesion was not blocked, and F-actin still accumulated at cell border, although the actin was less bundled. Our results reveal a major role for myosin in establishing supramolecular actomyosin arrays at cell junctions, and also suggest there maybe two independent pathways to generate F-actin pools at ECad-based junctions.

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Contraction and organization of cortical myosin minifilaments regulate the pattern of epithelial microridge protrusions
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Periodic patterns occur on many biological scales. Microridges are projections that form highly-organized, labyrinthine patterns on the apical surface of mucosal epithelial cells. Microridge form via the coalescence of finger-like precursor protrusions, a process that requires polarity establishment, Non-muscle Myosin II (NMII) contraction, Arp2/3 activity, Plakin cytolinkers, and keratins. However, little is known about how microridges adopt their distinctive, regular arrangement after they have formed. To investigate how microridges adopt highly-organized patterns, we imaged them in live, transgenic zebrafish larvae expressing the F-actin reporter Lifeact-GFP. Microridges had formed by 48 hpf, but between 48 and 96 hours post fertilization (hpf), their arrangement became more regular: the distance between adjacent microridges became less variable, and microridges became better aligned with their neighbors. Time-lapse imaging revealed that microridges dynamically rearrange, both breaking apart and joining together. Rapidly stretching cells by ablating their neighbors did not induce microridge rearrangement, suggesting that breaking and joining events do not simply result from deformation. Live imaging of Lifeact-mRuby and the NMII reporter Myl12.1-GFP revealed that microridge rearrangements spatially and temporally correlate with NMII contractions. To further investigate the spatial organization of NMII in the cortex, we used higher resolution Airyscan microscopy to image F-actin and NMII, which allowed us to detect individual NMII minifilaments in the cortex. Minifilaments were tethered to protrusions, often appearing to connect adjacent microridges. Live imaging showed that NMII minifilaments connecting the tips of microridges appeared to pull microridges together, whereas NMII minifilaments perpendicular to microridges appeared to either sever them or pull adjacent microridges closer together. These observations suggest that NMII minifilaments organize and rearrange microridges as their pattern matures. Indeed, inhibiting NMII contraction with the small molecule Blebbistatin blocked microridge rearrangement and dramatically altered microridge spacing. Collectively, our results demonstrate that the mature arrangement of microridge protrusions is influenced by their continuous rearrangement, which is driven by NMII contraction. These findings reveal a novel mechanism of subcellular pattern formation.

P250

Divalent ionic regulation for formation of actin, a microtubule and an actin-microtubule in vitro
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Actin and microtubules are main components of cytoskeleton, which are responsible for the cellular shape and motility through their dynamic interaction with their own unique morphological features. Although there are many known coupling proteins responsible for bridging actin and microtubules, little has been known how actin filaments and microtubules interact in a confined space while they are assembling. In order to understand the interaction between the two fibrils in the process of formation, the co-formation of actin-microtubules without any coupling protein was studied. By addition of divalent ions which are commonly required for the fibril formations of two proteins, we have confirmed how actin acts as a barrier to the growth of microtubules. By varying ion concentrations and protein concentrations, how the presence of one filament influences to the morphological features in the growth of the other. It has also been confirmed that actin filaments, by surrounding the microtubules, can participate in the "cooperation of membrane protrusion" mechanism, which serves to increase the strength of the microtubules in bulk. Moreover, we will also present some preliminary results of their co-formation dynamics in a confined space, a giant unilamellar vesicle.

P251

**Long Ventral Stress Fibers In Fibroblasts Form By Merging Of Short Actin Bundles At Adhesions.**

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Stress fibers are higher order cytoskeletal structures made of bundled actin that facilitate processes such as cellular force generation, migration and morphogenesis. Ventral Stress Fibers (VSFs) are contractile acto-myosin fibers present in the ventral plane of the cell that connect to the extra-cellular matrix through a focal adhesion on either end. Research on VSF dynamics have shown the presence of short fibers and longer fibers stretching across the cell. Our studies in lung fibroblast cells show that long VSFs can form by the merging of short fibers at intervening adhesions. This process involves formation of a myosin bridge between short merging fibers, along with efficient disassembly of the intervening adhesion, to form a single, longer, contractile VSF. Merging also orients the VSFs by aligning with the long axis of the cell, thus bearing functional significance in directional force production by fibroblasts and cell migration.

P252

**Ventral Stress Fibers Contour the Plasma Membrane in Human Fibroblasts**

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The interaction between the actin cytoskeleton and the plasma membrane is essential for many eukaryotic cellular processes including migration, endocytosis, and mechanotransduction. In these processes, actin fibers deform the cell membrane by applying forces at the ends of the fiber, either though actin fiber polymerization (“actin treadmilling”) or through myosin-mediated fiber contraction. Here we describe a novel actin-membrane interaction where actin stress fibers deform the plasma membrane along the length of the fiber. Briefly, human fibroblasts were treated with TGF-β to induce a fibroblast to myofibroblast transition and loaded with cytosolic dye or transfected with cytosolically-expressed fluorescent protein. After 96 hours, the cells developed prominent fluorescent ridges on the ventral side of the cell, visible via spinning disk confocal microscopy. Subsequent staining with
membrane dye, phalloidin (actin), and paxillin (focal adhesions) confirmed that the ridges were present in the cell membrane and colocalized with ventral actin stress fibers. Therefore, we hypothesized that ventral actin stress fibers are coupled to the membrane both at focal adhesions and along the length of the fiber. Furthermore, we hypothesized that these stress fibers induce membrane bending to form a “cytosolic pocket” for the cytosolic dyes to flow into, creating the observed ridges along the length of the stress fibers. Staining for the actin-membrane binding proteins ezrin/radixin/moesin confirmed that these proteins colocalize with the stress fibers, suggesting that the membrane can directly interact with the stress fiber. Subsequent treatment with cytochalsin D to dissolve the stress fibers removed the observed fluorescent ridges that colocalized with stress fibers, but not focal adhesions. As ridges associated with the intact focal adhesions remain, but ridges associated with the stress fibers disappear, this experiment suggests that membrane deformation is caused by proteins associated with stress fibers binding to the membrane. Lastly, we combined these results with computational modeling to estimate the necessary force generated by the actin fibers to bend the membrane. Taken together, these results demonstrate that actin stress fibers can deform the membrane in a ridge along their length in addition to deforming it at the ends of bundles through focal adhesions.

P253

Calponin-homology domain mediated bending of membrane associated actin filaments
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Actin filaments are central to numerous biological processes in all domains of life. Driven by the interplay with molecular motors, actin binding and actin modulating proteins, the actin cytoskeleton exhibits a variety of geometries. This includes structures with a curved geometry such as axon-stabilizing actin rings, actin cages around mitochondria and the cytokinetic actomyosin ring, which are generally assumed to be formed by short linear filaments held together by actin cross-linkers. However, whether individual actin filaments in these structures could be curved and how they may assume a curved geometry remains unknown. Here, we show that “curly”, a region from the IQGAP family of proteins from three different organisms, comprising the actin-binding calponin-homology domain and a C-terminal unstructured domain, stabilizes individual actin filaments in a curved geometry when anchored to lipid membranes. Whereas F-actin is semi-flexible with a persistence length of ~10 μm, binding of mobile curly within lipid membranes generates actin filament arcs and full rings of high curvature with radii below 1 μm. Higher rates of fully formed actin rings are observed in the presence of the actin-binding coiled-coil protein tropomyosin, and also when actin is directly polymerized on lipid membranes decorated with curly. Strikingly, curly induced actin filament rings contract upon the addition of muscle myosin II filaments and expression of curly in mammalian cells leads to highly curved actin structures in the cytoskeleton. Taken together, our work identifies a new mechanism to generate highly curved actin filaments, which opens a new range of possibilities to control actin filament geometries, that can be used, for example, in designing synthetic cytoskeletal structures.
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**Autophagy provides a cellular barrier against *E. coli* vector gene transfer in a mammalian cell.**

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The advent of the non-viral gene delivery systems is a crucial tool to realize a safe delivery without substantial side effect often elicited by the viral vector. Of the non-viral vectors, the bacteria-mediated vector is an attractive choice because of its low toxicity, immunogenicity and ability to deliver large DNA constructs both *in-vitro* and *in vivo*. *E. coli*-based vector utilizes exogenous genes such as invasin from *Yersinia pseudotuberculosis* and listeriolysin O from *Listeria monocytogenes*, to enable the transfer of expression plasmids into eukaryotic cells. However, the efficiency is limited by cellular obstacles which have been identified to include the internalization, endosomal escape and nuclear localization of the cytosolic DNA. Further, based on the understanding of the *E. coli* trafficking pathway, yet another potential barrier may exist. Here, we showed that the *E. coli* vector induced autophagy in mammalian cells, and this process is an intracellular efficiency-limiting process to gene transfer. Formation of LC3, an autophagosome marker, was observed in *E. coli* infected cells but not in Atg 5−/− cells. Further proof for autophagy was obtained by transfecting HeLa cells with *E. coli* vector, and this increased transgene expression up to 3-fold in atg 5−/− cells that are unable to generate autophagosomes. This work reveals that autophagy should be added to the list of cellular barriers to bacteria gene delivery into the mammalian cells, therefore providing a new design principle for targeted bacteria delivery vehicles.

P255

**Mechanisms that determine phagocytic appetite**

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Phagocytosis is the process by which cells like macrophages sequester and engulf external particles such as microbes within organelles called phagosomes. Phagosomes then fuse with lysosomes to become phagolysosomes, wherein its contents are degraded. This process is employed by macrophages to clear microbes during infections, as well as to eliminate apoptotic cells and other cellular debris. Macrophages can engulf dozens of particles. However, macrophages have finite resources that impose a limit onto their phagocytic capacity. Beyond this phagocytic capacity, macrophages need to “digest” the material within phagosomes to engulf again. Our goal is to determine the mechanisms and limiting factors that cause phagocytosis to halt at saturation. This could be broadly due to exhaustion of phagocytic membrane receptors, membrane levels, or increased internal membrane tension due to engulfed particulates. Using RAW 264.7 mouse macrophages and immunofluorescence against model phagocytic receptors like CD36, which engulf bacteria, we provide evidence that phagocytic receptors are not depleted at phagocytic saturation and surface receptors can still bind to bacteria when macrophages are “full”. Using indicators to observe downstream signaling, our future work will determine whether downstream receptor signaling is impaired at saturated phagocytic capacity. Interestingly, using fluorescence microscopy and the CellTracker cytoplasmic dye, our volumetric analyses show that macrophages' volumes at phagocytic saturation depend on the size and shape of the
particle engulfed. We speculate that this may cause increased internal pressure and membrane tension, which may halt additional phagocytosis when cells reach phagocytic saturation. Overall, while macrophages have a large phagocytic appetite, they do have a limit. Yet, very little is known about what imposes this limit, which our work will help address.

P256

Phagolysosome biogenesis novel regulators targeted by human rhinovirus infection
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Phagocytosis is a mechanism of internalization and degradation of micro-organisms or cellular debris. Phagocytosis is important for remodelling of tissues, disposal of dead cells and bacterial clearance. Once closed, the phagosome undergoes a series of fusion and fission with the endocytic compartments and evolves into a phagolysosomal compartment where degradation occurs. Human rhinovirus (HRV) is the most frequently isolated virus during severe exacerbations of chronic obstructive pulmonary disease in which alveolar macrophages are reported to display significantly diminished phagocytic and regulatory functions, associated with bacterial superinfections. However, how HRV affects the function of macrophages is largely unknown. Alveolar macrophages isolated from bronchoalveolar lavage or monocyte derived macrophages (MDMs) were pre-challenged with HRV16. Following viral challenge, we analysed phagosome maturation, reactive oxygen species generation, degradative activity and bacterial clearance. Alveolar macrophages or MDMs pre-challenged with HRV16 demonstrated deficient phagosome maturation and killing activity towards a range of targets. This inhibition was viral mediated and not observed with UV inactivated virus. We performed RNA sequencing on HRV16 infected macrophages in the presence or absence of a second bacterial challenge. We identified novel cellular regulators targeted by the human rhinovirus, allowing the virus to perturb phagolysosome biogenesis and bacterial clearance.

P257

Interactions between anaerobic ciliates and their prokaryotic symbionts
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Ciliates are a diverse group of ubiquitous unicellular eukaryotes that have successfully colonized environments considered extreme for the vast majority of eukaryotes, such as oxygen-depleted sediments. The evolutionary reasons for the prevalent anaerobiosis within ciliates are largely unknown, but the ability of ciliates to commonly form symbiotic relationships with a broad range of organisms may play a role. As a case in point, anaerobic ciliates harbor intracellular archaea that increase their host’s metabolism efficiency by utilizing hydrogen molecules, produced by the ciliate’s mitochondrial fermentation, to synthesize methane, one of the most potent green-house gases. Despite the global significance of archaea, the nature of their intracellular symbioses remains largely hidden and our knowledge on their host specificity, coevolution, and mode of the transmission is seriously limited to the
casuistic character of most studies. Furthermore, their occurrence within a host cell has been detected in only several lineages across the eukaryotic tree of life. To deepen our understanding of the relationship and interactions between methanogenic archaea and anaerobic ciliates, as well as other prokaryote-ciliate symbioses, we studied selected cultivated representatives of the main lineages of the recently determined deep anaerobic clade of ciliates (titled APM), in which we confirmed the presence of archaeal and bacterial symbionts by using fluorescence and transmission electron microscopy. We analyzed the 16S rRNA gene sequences of intracellular archaea and ectosymbiotic bacteria from selected APM ciliates from freshwater and marine environment. Apart from archaeal endosymbionts, we have also identified methanogenic archaeal ectosymbionts, which have not been previously described in a free-living organism. Assessing the host specificity and general intracellular interactions between anaerobic ciliates and their endosymbiotic methanogenic archaea, as well as their other prokaryotic symbionts, will enable us to further comprehend the origins of anaerobiosis in eukaryotes and their adaptations to life without oxygen. Moreover, understanding the unique symbioses between microbial eukaryotes and intracellular archaea is crucial to our perception of cell-cell interactions across domains of life.

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Differential Internalization of Rab11-Family Interacting Protein Vesicles into the Replicative Niche of Toxoplasma gondii

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Toxoplasma is an obligate, intracellular parasite that forms a parasitophorous vacuole (PV) upon invasion of mammalian cells. Since the PV is nonfusogenic, the PV membrane (PVM) impedes nutrient acquisition by the parasite. While smaller nutrients like free amino acids and simple sugars can enter through pores in the PVM, Toxoplasma has evolved specific mechanisms to access insoluble lipids. These processes are especially important since the parasite cannot synthesize all necessary lipids, like cholesterol, and therefore merit study to discover potential methods of therapeutic intervention (e.g., parasite starvation) with less side effects than current treatment. We have previously shown in mammalian cells transfected with fluorescently tagged Rab proteins and infected with Toxoplasma that, rather than fusing with host vesicles and organelles, Toxoplasma can internalize whole host cell vesicles from the endocytic, recycling, and exocytic pathways into its PV. To further investigate the selectivity in Rab vesicle hijacking, we focused on the Rab11a recycling endosomes, which still contain nutrients from the media and are very prevalently scavenged by Toxoplasma. We hypothesized that examining the Rab11 effectors called Rab11-Family Interacting Proteins (FIPs) would pinpoint where in the recycling endosome cycle Toxoplasma intercepts these vesicles. The FIPs are divided into two classes and all contain a conserved Rab11-binding domain. We found class I FIPs, FIP1C, FIP2, and FIP5, in 12%, 57%, and 52% of the PVs surveyed. For the class II FIPs, FIP3 and FIP4, we found them in 88% and 50% of PVs. As expected, mutating the Rab11-binding domain of the class I FIPs prevents their internalization into the PV, but surprisingly, the class II FIP Rab11-binding mutants were still present in 74% and 100% of PVs observed. This differential uptake leads to a distinction between Rab11-dependent and Rab11-independent internalization. To further analyze Rab11-independent internalization, we hypothesized that the class II FIP interaction with Arf6 mediates internalization in the absence of Rab11 recognition. In infected cells transfected with mCherry-tagged Arf6, we observed Arf6-containing vesicles within the PV.
Our preliminary experiments with mutating both class II FIP3 Rab11-binding and Arf-binding domains show no internalization of FIP3, thereby allowing us to designate Rab11-independent internalization as Arf-dependent internalization. These studies highlight two separate processes of host vesicle internalization into the PV, strongly suggesting that Toxoplasma expresses potentially drug-targetable proteins at the PVM that selectively recognize host organelles and vesicles and that are important for Toxoplasma essential lipid acquisition.

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*Chlamydia trachomatis* alters p53 in epithelial cells and manipulates host cell lipid metabolism and CD40 signalling polarizing anti-inflammatory macrophages


*Chlamydia trachomatis* repeated/latent infection is responsible for ectopic pregnancies, abortions, pelvic inflammatory disorders and infertility. During infection, T cells produce IFN-γ which depletes IDO and activates CD8⁺T cell to clear Ct. The study is aimed at understanding persistent chlamydial infection and role of macrophage polarisation in it. We observed that low IFN-γ decreases the bacterial burden but could not clear the bacteria. Infected epithelial cells Co-cultured with macrophages, skewed macrophages(mφ) to alternative activated mφ (AA-mφ) with increased CD163 and arginase levels and decreased iNOS levels. Further, macrophages infected with Ct secreted high IL-1β, TNF-α along with increased IL-10 production. On co-culture of infected mφ with Naïve T cells, T cells secreted both inflammatory INF-γ and anti-inflammatory: IL-4, IL-10 and TGFβ. INF-γ mediated persistent infection in mφ showed increased IL-10 production and differentiated naïve T cells to high IL-4 and IL-10 producing cells. These mφ also showed decreased CD40 expression and increased PDL-1. Ct infected macrophages also show altered lipid metabolism which might favour AA-mφ differentiation. Ct infected macrophages show high phosphatidylethanolamine and ceramides and display similar lipid metabolites to M2 macrophages. Ct also caused p53 mutations leading to altered isoforms which plays a role in inflammation. Furthermore, AA mφ on infection display higher bacterial load and potentiate IL-10 production compared normal and CA mφ on infection and varied expressions of PDI and Ephrin B2. On co-culture of Naive T cells with differentially polarised infected mφ, AA mφ differentiated T cells towards high IL-4 and IL-10 producing cells. T cell cytokines: IFN-γ could decrease the bacterial burden, while IL-4 did not show any change and IL-10 increased bacterial burden. Ct infection of BALB/c mice elicits IFN-γ producing Ct-specific T cells and INF-γ in cervical lavage. While, repeated Ct infection decreased INF-γ in cervical lavage and Ct-specific T cell proliferation with low INF-γ and increased IL-4 secretion. T cell anergy, senescence and exhaustion have been explored to analyse the decreased T cell proliferation. In conclusion, Ct manipulates lipid metabolism, mutate p53 leading to varied CD40 expression which are involved in AA mφ skewing in turn decreasing IFN-γ producing T cells to sustain its growth to propagate for longer duration. Keywords: Chlamydia trachomatis, Macrophage polarisation, P53 isoforms, persistent infection, inflammation.
Nramp1 at the interface of battle for iron between *Leishmania* and its host macrophage

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The proliferation of the protozoan parasites, *Leishmania* which infect and survive within the phagolysosomal compartment of mammalian macrophages is critically dependent on iron. The underlying mechanism by which *Leishmania* acquires iron from its iron-limiting intracellular niche is still not clear. Natural resistance associated macrophage protein 1 (Nramp1) is an iron transporter which was originally discovered as a resistance-determining gene against multiple intracellular pathogens including *Leishmania*. However, the exact mechanism by which functioning of Nramp1 determines the pathogenesis of *Leishmania* is yet to be discovered. Hence, the current objective is to understand how *Leishmania* modulates macrophage Nramp1 to obtain iron within phagolysosomal compartment. To evaluate this, J774A.1 and peritoneal macrophages of BALB/c mice has been infected with *Leishmania major* (*L. major*) and Nramp1 expression was determined using Western blot and qRT-PCR and compared between uninfected and infected cells in a time dependent manner. Ferrozine based assay was performed to quantitate intracellular and phagosomal iron content of uninfected and infected macrophage cells. Moreover, pharmacological inhibition study and co-immunoprecipitation has been performed to check whether degradation of Nramp1 follows ubiquitin-proteasomal pathway during *L. major* infection. Additionally, involvement of the iron regulatory peptide hormone, hepcidin in determining Nramp1 expression has been deciphered using immunofluorescence, qRT-PCR and pharmacological inhibitor studies. Our results show infection of macrophage cells with *L. major* significantly reduces Nramp1 protein expression at 12hrs post infection accompanied by elevated intracellular as well as phagosomal iron content of macrophages which in turn stimulates the replication of the parasites. However, restoration of Nramp1 to normalcy at 30hrs post infection with a concomitant drop in phagosomal iron suggests host counteractive response against the parasite to check its growth by limiting the availability of this essential micronutrient. Importantly, we have established that degradation of Nramp1 during infection follows the ubiquitin-proteasomal pathway which was found to be mediated by the iron-regulatory peptide hormone hepcidin. Blocking of Nramp1 degradation with proteasome inhibitor or transcriptional agonist of hepcidin resulted in depletion of phagolysosomal iron pool that led to significant reduction of intracellular parasite burden. Collectively, our study for the first time provides direct evidences of Nramp1 to be a central player in the host-pathogen battle for phagolysosomal iron by unraveling a novel ‘hepcidin- Nramp1’ axis in context to macrophage iron homeostasis.

**Microtubule Dynamics**

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Microtubule minus-end stability is dictated by the tubulin off-rate

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Dynamic organization of microtubule minus ends is vital for the formation and maintenance of acentrosomal microtubule arrays. In vitro, both microtubule ends switch between phases of assembly and disassembly, a behavior called dynamic instability. Although minus ends grow slower, their lifetimes are similar to those of plus ends. The mechanisms underlying these distinct dynamics remain unknown. Here, we use an in vitro reconstitution approach to investigate minus-end dynamics. We find that minus-end lifetimes are not defined by the mean size of the protective GTP-tubulin cap. Rather, we conclude that the distinct tubulin off-rate is the primary determinant of the difference between plus- and minus-end dynamics. Further, our results show that the minus-end-directed kinesin-14 HSET/KIFC1 suppresses tubulin off-rate to specifically suppress minus-end catastrophe. HSET maintains its protective minus-end activity even when challenged by a known microtubule depolymerase, kinesin-13 MCAK. Our results provide novel insight into the mechanisms of minus-end dynamics, essential for our understanding of microtubule minus-end regulation in cells.

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ATAXIN-2 is an essential regulator of cytoskeletal dynamics in Drosophila neurons

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Ataxin-2 (Atx2) is a highly conserved RNA binding protein. Atx2 undergoes polyglutamine expansion leading to amyotrophic lateral sclerosis (ALS) or spinocerebellar ataxia (SCA). However, the normal physiological functions of Atx2 remain unknown, likely because of functional redundancy between Atx2 and Atx2-like genes in mammals. Here we use the powerful genetics of Drosophila, that have only a single Atx2 gene, to show that Atx2 is essential for normal cytoskeletal dynamics and for neurodevelopment. We depleted Atx2 specifically in neurons and found severe impairments in cytoskeletal dynamics both in microtubule and actin networks. Microtubules became hyper-stabilized, as demonstrated by increased acetylation and resistance to microtubule depolymerising drugs, and kinesin-driven microtubule-microtubule sliding was inhibited. Similarly, we found F-actin was hyper-stabilized as shown by resistance to actin depolymerising drugs. Further, in neurons both in vitro and in vivo we found that transport of multiple cargoes was severely decreased. Beyond the defects in the cytoskeleton, we found neuron-specific depletion of Atx2 caused many morphological defects in the nervous system of third instar larvae. These include reduced brain size, impairments in optic lobe innervation and decreased dendritic arborization in sensory neurons. Defects in the nervous system of these larvae caused loss of the ability to crawl and were lethal at the pupal stage. Taken together, these data mark Atx2 as a master regulator of the cytoskeleton and denote Atx2 as an essential gene in neurodevelopment, as well as a neurodegenerative factor. These data will provide insight into therapeutic interventions for Atx2 polyglutamine disorders.

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Cytoplasm properties limit cytoskeleton dynamics

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The cytoplasm is a very crowded environment harboring most of the metabolic reactions necessary for cellular function. Its biophysical properties are influenced by cellular metabolism. Indeed, changes in cytoplasm mechanical properties have been linked to cellular differentiation and changes in cytoplasm concentration to cell death. However, the reverse relationship has not been extensively studied. So, here we tried to address the effects of the cytoplasm biophysical properties on various dynamic processes happening inside cells from protein diffusion to cytoskeleton dynamics. Our results demonstrate the sensitivity of cellular dynamics, specifically of the cytoskeleton dynamics, to cytoplasm concentration. In fission yeast, changing cytoplasmic density reversibly affects all the dynamic processes we looked at from protein diffusion to complex dynamic processes such as actin and microtubule dynamics. We found that microtubule dynamic is inversely correlated to cytoplasmic density. Increasing cytoplasm density slowed down microtubule growth and shrinkage rates in a dose-dependent. This effect of cytoplasm density on cytoskeleton dynamics did not seem to depend on stress pathway. We extended our observation to other eukaryotes, mammalian, and plant cells, and confirmed the effect of cytoplasm density on microtubule dynamics suggesting that we are probing an inherent property of the cytoplasm. We discovered that the effect of cytoplasmic density on the cytoskeleton can be alleviated and that a dilution of the cytoplasm leads to an increase in microtubule dynamics even if tubulin concentration decreases. We set out to explore the effect of the environment biophysical properties on microtubule dynamics in vitro by growing microtubule in buffers of various viscosity and obtained supporting evidence. Our results highlight how important the biophysical properties of the cytoplasm are to cellular metabolism in eukaryotes and suggest that the viscosity of the cytoplasm limits how fast processes such a cytoskeleton dynamic can happen in cells. This study reveals the interplay between biology and physics happening in the cytoplasm. Our assay is a first step in understanding how the biophysical properties of the cellular environment affect the biological processes it encloses.

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Microtubules Targeting Agents Inhibit Wnt/β-Catenin Signaling by Inhibiting the Nuclear Translocation of β-Catenin in Oral Squamous Cell Carcinoma


Wnt/β-catenin signaling pathway is up-regulated in various types of cancers and the augmented activity of Wnt/β-catenin signaling is known to increase the stability of microtubules. In this work, we examined the interplay between microtubule stabilization and Wnt/β-catenin signaling using small molecule inhibitors of microtubules. Here, we report that a microtubule-depolymerizing agent C12 (5-Quinolin-3-yl and 4-(3,4,5-trimethoxyphenyl) substituted imidazol-2-amine) inhibits the proliferation of oral squamous cell carcinoma (H357) cells and also potently inhibit tumor progression on the tongue of black 6 mice. Like other microtubule-targeting agents, C12 arrests oral cancer cells in mitosis and induces apoptosis in both cell culture and mice models. Interestingly, C12 treatment reduced Wnt/β-catenin signaling both in vitro and in vivo. Like C12, vinblastine, and taxol were also found to inhibit Wnt/β-catenin signaling in H357 cells by reducing the levels of positive regulators of Wnt/β-catenin signaling like β catenin, and disheveled-1 while increasing the levels of negative regulators like APC and Axin-1. Further, the level of nuclear β-catenin was reduced in the presence of both microtubule-stabilizing
(taxol) and microtubule-destabilizing (C12 and vinblastine) agents indicating that microtubule dynamics may play important role in nuclear localization of β-catenin. Also, we found evidence indicating that kinesin 2 is responsible for the translocation of β-catenin. We also checked the efficacy of anti-microtubule agents in the presence of antagonists and agonists of Wnt/β-catenin signaling. The results show that the microtubule stability and dynamics and Wnt/β-catenin signaling are closely interlinked.

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**CLASPs depolymerize microtubules in a nucleotide-dependent manner**

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CLASPs belong to a family of evolutionarily-conserved TOG-domain proteins that regulate microtubules in fundamental cellular processes including cell division, cell migration and neuronal development. Another TOG-domain protein, XMAP215/chTOG, promotes microtubule polymerization in the presence of soluble tubulin but depolymerizes microtubules in the absence of soluble tubulin. In contrast, CLASPs stabilize dynamically growing microtubules by suppressing catastrophe and promoting rescue, the switch-like transitions between growth and shrinkage. Previous work found that the isolated TOG2 domain of CLASP2 is sufficient for recapitulating the core activity of the full-length protein in vitro. However, the molecular mechanisms underlying CLASP’s activity are not fully understood. Here, we report the surprising finding that human CLASP1 depolymerizes stabilized microtubules in the presence of GTP and GDP but not in the presence of GMPCPP nor in the no nucleotide condition. Thus, CLASP1 depolymerizes stable microtubules in a nucleotide-dependent manner. Further analyses revealed that other members of the human CLASP family (CLASP2α and CLASP2γ) also possess nucleotide-dependent depolymerase activity, as does the minimal TOG2-domain construct. Next, we demonstrate that CLASP1 depolymerizes both plus and minus microtubule ends in a nucleotide-dependent manner; this is unexpected, given that the exchangeable nucleotide site in tubulin is presumed to be buried within the polymer at the minus end. By performing single molecule dwell time analysis, we find that GTP reduces the dwell time of TOG2 at microtubule tips compared to the no nucleotide condition. Finally, we report that the depolymerase activity of CLASP1 exhibits significantly greater sensitivity to GTP than that of XMAP215/chTOG, indicating that the two TOG-domain proteins employ distinct mechanisms to depolymerize microtubules. The unanticipated finding that CLASPs possesses nucleotide-sensitive depolymerase activity provides critical mechanistic insights into the activity of an important family of microtubule regulatory proteins. This work will further our understanding of the mechanisms employed by CLASPs in regulating microtubule network dynamics and organization in cells.

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**Kinesin-14 motors participate in a force balance at microtubule plus-ends to regulate dynamic instability**

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Kinesin-14 molecular motors represent a surprising and highly conserved class of Kinesin motors that bind microtubules and walk towards their minus-ends. Previous studies have elucidated important roles for Kinesin-14 motors in stabilizing and organizing microtubule minus-ends, but the role of Kinesin-14
motors in regulating microtubule plus-end dynamics remains controversial. Kinesin-14 motor proteins have been shown to bind the EB family of microtubule plus-end binding proteins, suggesting that minus-end directed Kinesin-14 motors could interact with growing microtubule plus-ends via EB proteins. In this work, we explored the role of a force balance between microtubule polymerization forces, and minus-end directed Kinesin-14 motor forces, in controlling plus-end microtubule dynamics. To dissect this force balance and its effect on microtubule dynamics, cell-free biophysical TIRF microscopy assays were performed using fluorescently labeled dynamic microtubules, minus-end directed kinesin motors (Kinesin-14-GFP), and an EB-family marker of growing microtubule plus-ends, Mal3-mCherry. We found that when Kinesin-14 motors bound to the Mal3 tip tracker at growing microtubule plus-ends, the motors subsequently walked towards the minus-end, pulling Mal3 away from the microtubule tip. Strikingly, these interactions resulted in catastrophe events 52% more quickly than in experiments with mutant motors that did not bind Mal3 (p=0.03, t-test). In contrast, loss of Kinesin-14 motility under rigor conditions led to prolonged tip interactions at growing microtubule plus-ends, without a catastrophe event (interaction time as fraction of total growth time: 0.52±0.06 for HSET in AMPPPNP; 0.035±0.003 for HSET in ATP, p<<<0.0001; mean±SEM). Imaging data as well as computational modeling led us to hypothesize that active Kinesin-14 motors, when bound to Mal3, exert minus-end directed forces to pull back on protofilament(s) at the plus-end, disrupting the growing microtubule tip structure, and rapidly causing catastrophe. In contrast, Kinesin-14 motors in rigor conditions (without minus-end motility) track the growing microtubule end in a plus-end direction, suggesting that plus-end directed polymerization forces remain predominant. Together, our data demonstrate that Kinesin-14 motors participate in a force balance at microtubule plus-ends to regulate dynamic instability.

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Astral microtubule repulsion drives nuclear packing and ordering in the Drosophila blastocyst

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The fertilised insect egg undergoes a series of rapid nuclear divisions without cleavage of the cell membrane, generating a multinucleated blastoderm embryo. In Drosophila melanogaster, the embryo develops by dividing nuclei synchronously every ~9 minutes for nine cycles after which most of the nuclei reach the cell cortex. Nuclear division and transport processes depend on microtubules, microtubule organising centres (MTOCs) and f-actin. Despite the rapid nuclear division and repositioning, the spatial pattern of nuclei on the cortex is highly regular. Such precision is important for subsequent cellularisation and germ band formation. We investigated the mechanical interactions between MTOCs that lead to ordered distribution of dividing syncytial nuclei. We show in embryo mutants and explants that microtubule asters are necessary and sufficient for regular distribution maintenance in the embryo. For large networks of nuclei, such as in the embryo, we predict - and experimentally verify - the formation of force chains. Utilising ex vivo extracts, we infer the nature of the force potential between asters. We use this to predict how the nuclei division axis orientation in small ex vivo systems depends on aster number. Finally, we demonstrate using laser ablation that the microtubule force potentials are able to reorient subsequent nuclear divisions, such that the size of nuclear pattern defects are minimised. Overall, we show that short-ranged microtubule-mediated repulsive interactions between MTOCs contribute to precise nuclear positioning during early Drosophila development.
Microtubule pushing forces contribute to long-distance aster centration in *Xenopus laevis* egg extracts

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The cytoplasm of a eukaryotic cell is a complex and dynamic microenvironment that requires proper organization to insure cell function and homeostasis. The microtubule (MT) cytoskeleton is critical in this organization. During interphase, MTs serve as both a supportive scaffold for organelles and as an arborized system of tracks for intracellular transport. During mitosis, the position of the interphase MT aster determines the eventual location of the spindle apparatus and ultimately the cytokinetic furrow. Asters position is reliably established in center of a cell, even in large blastomeres hundreds of microns in diameter and in unnaturally imposed cell geometries like squares and rectangles. This positioning requires the generation of MT based forces and in this context there are thought to be three primary forces: (i) MT pushing forces generated by the interaction between growing MT plus ends and the cell cortex, (ii) cortical pulling forces produced by minus end directed motors (dynein) anchored at the cell cortex, and (iii) cytoplasmic pulling forces generated by dynein-mediated transport of vesicular cargo through the viscous cytoplasm. How these different forces are integrated to position asters within cells of varied sizes, geometries, and in different systems remains an open question. To address it, we have developed the use of microfluidic devices and photolabile hydrogels with *Xenopus laevis* egg extract to capture and release artificial microtubule organizing centers (aMTOCs) in geometrically defined hydrogel microenvironments. Using this approach, we found that interphase asters are able to center themselves along the short-axes of channels, ~100 µm in width, and in annular cylindrical enclosures of ~100 µm in diameter. Importantly, inhibition of cytoplasmic dynein, with p150-CC1, did not affect aster centration over the length scales explored in these studies. When aster formed in asymmetric structures, the aMTOCs would be de-centered in a manner consistent with the pushing model. Characterizations of aMTOC movement away from open-ended V-shaped hydrogel barriers, purposefully designed to exaggerate aster asymmetry, were inconsistent with pulling-based mechanisms. In sum, this work suggests that aster MTs are able to exert pushing forces that play a primary role in aster movement in egg extract, at least over the length scales investigated.

New Techniques in Cell Biology

A modular tool to query and inducibly disrupt biomolecular condensates

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Biomolecular condensates are dynamic membraneless compartments in which multivalent proteins or proteins with intrinsically disordered regions (IDRs) undergo a phase transition that unmix the interior of the cell, imposing spatial and temporal organization. Tools that inducibly trigger condensate formation are useful for exploring their cellular function and utility, however there are few tools to induce condensate disruption. Here, we describe a new synthetic tool named DisCo (Disassembly of
Condensates), allowing conditional disruption of biomolecular condensates. We demonstrate use of DisCo to disrupt condensates relevant to neurodegenerative disease, comprised of the RNA binding protein FUS or huntingtin exon 1. Extending this approach, we combined DisCo with an optogenetic tool based on Arabidopsis cryptochrome 2, CRY2 olig, that forms condensates upon illumination. The combined use of these tools enables bidirectional control of condensate formation and disassembly using orthogonal inputs. We envision this new tool will provide insight into condensate disruption mechanisms, and facilitate further studies of the biological role of biomolecular condensates in a variety of model systems.

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Development of a Ribozyme-based Selection Mechanism for Metabolic Engineering in Microfluidic Droplets
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Our goal is to develop a new approach to the optimization of gene expression in support of metabolic engineering for applications in medicine, biofuels, chemical commodities, and the environment. Our plan is to construct a system by which the combinations of promoters and RBSs controlling the expression of metabolic enzymes that produce the most of a desired metabolite can be automatically selected for. Our prototype metabolic pathway is the conversion of caffeine to theophylline by caffeine demethylase (CDM). Theophylline is a broncho-dilator and the active ingredient in most asthma medications. We plan to introduce a library of DNA constructs that vary in promoter and RBS strengths into microfluidic droplets along with the materials needed for Recombinase Polymerase Amplification (RPA) and for CFPS (Cell Free Protein Synthesis). RPA is needed to produce copies of DNA constructs in droplets that can later be subjected to selection. RPA can be used to amplify DNA in just twenty minutes at room temperature. Reports in the literature indicate that RPA can amplify a template of at most 500 bp, but we have successfully used RPA to amplify a 1500 bp template. CFPS avoids concerns about applications that produce metabolites that interfere with the functions of a living cell. Our selection scheme is to use theophylline responsive ribozymes to transduce the theophylline levels in individual droplet into fitness. Our plan is to sandwich ribozyme RNA between two pieces of DNA through ligation. One piece of DNA with a thiol modified end will enable us to connect the ribozymes to amino modified magnetic nanoparticles (MNPs), and the other piece of DNA is the forward primer needed for RPA. Droplets in which high levels of theophylline are produced will have CDM constructs cleaved from MNPs, and we will use a magnet to separate out uncleaved constructs. We explored three theophylline ribozymes known to function well in bacterial cells and one that functions well outside of cells. We successfully cloned all four ribozymes and made two mutants of each. So far, we have demonstrated theophylline dependent cleavage of one of the four ribozymes. Soon we will be in a position to assemble the selection mechanism and test it in droplets.

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Investigation of pH-dependent cell behaviors with Archaerhodopsin, a spatiotemporal optogenetic tool for the manipulation of intracellular pH
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Intracellular pH (pHi) is tightly regulated in normal cells (7.0-7.2) and increases in pHi (7.4-7.6) are linked to a wide variety of pH-dependent cell behaviors including directed cell migration, cell polarization, actin remodeling, and cell cycle progression. Dysregulated pHi has been linked to diseases including cancer, where pHi is constitutively increased and enables increased invasion, metastasis, proliferation, and metabolic adaptation. However, most studies linking increased pHi to these normal and cancer cell behaviors have been performed at the population level using non-specific pHi manipulation tools. There is a need in the cancer and cell biology fields to understand how pHi dynamics regulate pH-dependent cell behaviors at the single-cell level. In this work, we present the characterization and use of Archaerhodopsin (ArchT), a light-activated outward proton pump, as a spatiotemporally-controlled optogenetic tool to manipulate pHi in single cells. We show that ArchT can be used to selectively and robustly raise pHi within single cells on the seconds to minutes time scale. We show that under a variety of experimental conditions, ArchT-mediated increases in pHi are robust, repeatable, and can be maintained within a physiologically relevant range for up to 40 minutes. We applied this tool to investigate the relationship between increased pHi and actin remodeling within single living-cells. For the first time, we show that single cells respond to a localized increase in pHi by undergoing spatially-restricted cytoskeleton remodeling and membrane protrusion. Localized membrane protrusion requires both ArchT expression and photoactivation, and sustained ArchT-mediated pHi increases can lead to retraction of the opposite (non-stimulated) side of the cell. Overall, this data suggests that ArchT can be used to investigate critical pH-dependent cell behaviors that were previously inaccessible due to limitations of current pH manipulation tools.

Modeling Stochastic Behavior of Size Control in the Multiple Fission Cell Cycle of Chlamydomonas

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Populations of proliferating cells display highly deterministic and predictable size phenotypes while individual cells show stochastic variation in growth and division. Understanding the sources of stochastic variation and how they impact size homeostasis remains a challenge. The unicellular alga, Chlamydomonas reinhardtii (Chlamydomonas), proliferates using a multiple fission cell cycle where growth and division are partly uncoupled. After a long G1 phase of indeterminate growth, mother cells undergo a rapid sequence of cell divisions (several rounds of alternating S and M phases) to produce daughter populations of uniform and reproducible size. Mitotic size control couples mother cell size to division number such that larger mother cells divide more times than smaller mother cells. We investigated and modeled the contributions of stochastic behavior to mitotic cell size control in Chlamydomonas using a microscopy-based single cell tracking assay applied to thousands of cells. We found that, unlike the case in yeasts and bacteria, the size distribution of Chlamydomonas daughter cells was invariant across a range growth rates, nutrient conditions, and pre-division mother-cell sizes, meaning there is a fixed target size-range for daughters. Despite this fixed target size, individual mother cells often fell short of or exceeded the optimal number of divisions required to produce daughters within the target size-range. Paradoxically, division behavior within an individual mother cell was
invariant: if after the first division, one daughter divided a second or third time, the other daughter did the same. The simplest and most intuitive model to explain population-level mitotic size control involves a noisy power law where mother cells within size ranges bounded by increments of $2^n$ divide $n$ times. Despite its simplicity and ability to generate empirically observed daughter cell-size distributions, the noisy power law model was a relatively poor predictor of individual mother cell division behavior. Instead we found a best fit to a model where the size-bounds governing division number are more constrained than the power law model in smaller mother cells and become gradually less constrained as mother cell size increases. This allometric scaling model, and the observation of invariant division behavior within individual mother cells, has implications for molecular mechanisms that govern mitotic size control in Chlamydomonas and possibly for other eukaryotes that have non-canonical cell cycles where growth and division are uncoupled. Our ongoing work involves testing the allometric model in cell-size mutants and identifying and testing potential sizer molecules that fit the behavior predicted by our allometric scaling model.

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Click chemistry-based bioorthogonal labeling of the axon initial segment in live primary neurons
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The axon initial segment (AIS) and the nodes of Ranvier are responsible for initiation and propagation of action potentials. These highly specialized neuronal subdomains are composed of membrane proteins, such as voltage-gated ion channels and neural cell adhesion molecules, that are linked to underlying cytoskeleton network via scaffolding protein ankyrin G. The unique role of the AIS and the nodes of Ranvier relies on high-density accumulation of voltage-gated sodium channels (Naₙ). Naₙ clustering is facilitated by a neural cell adhesion molecule - 186 KDa neurofascin (NF186). NF186 has a central role in formation of the nodes of Ranvier, and stabilization and maintenance of the AIS and the nodes. Additionally, NF186 inhibits axon outgrowth. Mutations of both Naₙ and NF186 have been implicated in various neurological disorders, while the antibodies against NF have been found in demyelinating diseases. Despite their importance, live imaging of the AIS is challenging due to the lack of suitable labeling methods. It has been reported that widely used fluorescent protein-based tags owing to their size can induce mislocalization and alter interactions of the AIS components. To overcome these limitations, we are developing a method for live labelling of the AIS with unnatural amino acid (UAA)-based minimal tags. UAAs carrying strained alkenes are site-specifically incorporated into target proteins by genetic code expansion technology and subsequently labeled with tetrazine dye derivatives in a bioorthogonal type of click-chemistry reaction (strain-promoted inverse-electron demand Diels-Alder cycloaddition). Our preliminary results show that NF186 can be labeled with click-chemistry in live neuronal cell lines and primary neurons. Currently, we are establishing labeling of other AIS components, such as Naₙ1.6 isoform. The small size of the UAA, and the possibility to introduce it virtually anywhere into the target proteins makes this method particularly suitable for live labeling and imaging of the AIS components. Further optimizations will allow us to follow dynamic changes happening in the AIS with conventional and super-resolution microscopy.
Minimal fluorescent tags for live-cell labeling of cytoskeletal proteins in primary neurons
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Neurofilaments are neuron-specific cytoskeletal elements, composed of three major neurofilament isoforms including neurofilament light (NfL), medium (NfM), and heavy (NfH) chains. They form intermediate filaments with a diameter of 10 nm and play important roles in maintaining axonal radial growth and diameter, and in the modulation of synaptic activity and plasticity. Live-cell labeling of NfM and NfH is commonly achieved by making fluorescent protein fusions. However, this approach is not optimal for labeling of NfL, as the attachment of relatively large fluorescent protein-based tags might affect its assembly in the neurofilament network. Consequently, there are very few live-cell imaging studies of NfL. To tackle this, we aimed to label NfL with a novel method, based on the combination of genetic code expansion (GCE) and bioorthogonal click-chemistry reactions. The main advantages of this method are its compatibility with live-cell labeling and the minimal size of the installed fluorescent tag. GCE utilizes an orthogonal translational machinery for the site-specific incorporation of an unnatural amino acid (UAA) at a desired position in the protein of interest. Incorporated UAA carries strained alkene or alkyne moiety that is subsequently conjugated to a tetrazine-bearing fluorophore via SPIEDAC (strain-promoted inverse-electron-demand Diels-Alder cycloaddition) click-chemistry reaction. The combination of GCE and click-reactions has been previously used for protein labeling in standard mammalian cell lines, but it has never been applied in complex cells, such as primary neurons. As will be presented here, we established this method for protein labeling in a neuronal cell line and primary neurons. With our approach, we labeled NfL in live neurons and imaged it both after fixation and in living cells. Moreover, we labeled two populations of NfL during neuronal growth in vitro. Finally, we applied this labeling method for super-resolution imaging of NfL. Our results demonstrate for the first time that the combination of GCE and bioorthogonal click-reactions can be employed for protein labeling in live primary neurons. We applied this method for the labeling of NfL and combined it with conventional and super-resolution microscopy. Additional optimizations will allow tracking NfL dynamics and transport, as well as super-resolution imaging in live neurons. In general, UAA-based minimal tags can be applied for the labeling of other neuronal proteins, providing a good alternative to the conventional fluorescent labeling tags.

Adhesion Strength of Axonal Growth Cone and Its Contribution in Axon Outgrowth Evaluated by Femtosecond Laser Impulse
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The process of axon outgrowth is regulated by retrograde flow of filamentous actin (F-actin) with specific reversible binding between the F-actin and complementary ligands on the substrate through cell
adhesion molecules. In the study, we used femtosecond laser-induced impulse as a tool to evaluate the adhesion strength between the cell adhesion molecules and ligands. An intense femtosecond laser was focused near the axon growth cone to generate an impulsive force with a micrometer scale explosion. The impulsive force to break adhesion between the growth cone and substrate increased with laminin density on the substrate, though such dependence was not observed in L1 cell adhesion molecule (L1-CAM) knockdown condition. These results indicate that adhesion strength between the growth cone and the substrate increases with the laminin density on the substrate. The adhesion strength would be related with the number of molecular clutches between F-actin and the L1-CAM, which regulate the F-actin retrograde flow. The retrograde flow in the axon was observed by fluorescence speckle microscopy and confirmed by tracking single molecules of F-actin and L1-CAM. The retrograde flow was slower by increasing the laminin density. The slowdown generates traction force to induce axon elongation, but the suitable elongation was not induced in excessive adhesion between the laminin and L1-CAM molecules. We showed contribution of the adhesion in F-actin retrograde flow to promote the axon outgrowth.

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Pilot evaluation of an enzymatic assay for rapid measurement of antiretroviral drug concentrations


Objective: Maintaining adequate drug adherence is crucial to ensure the HIV prevention benefits of pre-exposure prophylaxis (PrEP). We developed an enzymatic assay for rapidly measuring tenofovir-diphosphate (TFV-DP) concentrations - a metabolite that indicates long-term PrEP adherence. Setting: The study was conducted at the Madison HIV Clinic at Harborview Medical Center in Seattle. Methods: We enrolled adults receiving standard oral PrEP, and individuals not receiving any antiretrovirals. We measured TFV-DP concentrations in diluted whole blood using our novel REverSe TRanscrIptase Chain Termination (RESTRICT) assay, based on inhibition of HIV reverse transcriptase (RT) enzyme. Blood samples were diluted in water, DNA templates, nucleotides, RT, and intercalating dye added, and results measured with a fluorescence reader—stronger fluorescence indicated higher RT activity. We compared RESTRICT assay results to TFV-DP concentrations from matched dried blood spot samples measured by liquid chromatography tandem mass spectrometry (LC-MS/MS) using ≥700 fmol/punch TFV-DP as a threshold for adequate adherence (≥4 doses/week). Results: Among 18 adults enrolled, 4 of 7 participants receiving PrEP had TFV-DP levels ≥700 fmol/punch by LC-MS/MS. RESTRICT fluorescence correlated with LC-MS/MS measurements ($r=0.845$, $p<0.0001$). Median fluorescence was 93.3 (95% CI: 90.9 to 114) for samples <700 fmol/punch and 54.4 (95% CI: 38.0 to 72.0) for samples ≥700 fmol/punch. When calibrated to an a priori defined threshold of 82.7, RESTRICT distinguished both groups with 100% sensitivity and 92.9% specificity. Conclusions: This novel enzymatic assay for measuring HIV reverse transcriptase activity may be suitable for distinguishing TFV-DP concentrations in blood that correspond to protective PrEP adherence.
Uncovering unconventional conservation in meiotic proteins
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The Synaptonemal Complex (SC) is a structure that is instrumental to eukaryotic sexual reproduction. The SC ensures that offspring inherit the correct number of chromosomes by aligning parental chromosome pairs and regulating exchanges between them. In electron micrographs, the SC ultrastructure appears between chromosomes as a ~100 nm-wide ladder-like assembly with regular striations. Despite the essential, conserved function of the SC and its conserved dimensions and ultrastructure, the proteins that make up the SC are highly divergent. We took an evolutionary genomics approach to address this paradox. Specifically, we asked 1) What evolutionary forces explain the high divergence of SC proteins? and, 2) Are there conserved features of SC proteins that could explain their conserved function and ultrastructure? We found that, within Caenorhabditis, SC proteins are significantly more diverged than other proteins, but do not evolve under positive selection. This suggests that the high divergence of SC proteins can be explained by lack of constraint on the primary amino acid sequence. We hypothesized that there might be other conserved features of SC proteins including overall protein length and the length and positioning of predicted coiled-coil domains. To test this, we developed a bioinformatics pipeline that measures coefficient of variation of protein length as well as a coiled-coil conservation score for all proteins in Caenorhabditis. We found that SC proteins are highly conserved in both categories despite their amino acid divergence. This suggests that, in contrast to the amino acid sequence, the physical length and domain structure of SC proteins evolve under strong purifying selection and may be important for SC structure and function. Finally, our analyses indicate that SC proteins have a unique evolutionary signature of high amino acid divergence, but strict conservation of protein length and coiled-coil domains. We reasoned that this signature could be used to identify SC proteins in genome or transcriptome datasets even when primary amino acid sequence has diverged beyond the reaches of conventional alignment search algorithms such as BLAST. As a test scenario, we analyzed all proteins from 30 Drosophila species and were able to identify the known Drosophila SC proteins. Thus, our pipeline could be used to identify SC proteins without laborious genetic screens in emerging model systems where genome data are plentiful. Indeed, we have applied our pipeline to Aedes and Anopheles mosquitoes, where the SC proteins are currently unknown, and have a short list of candidate SC proteins. Overall, this study sheds light on important conserved features of SC proteins and provides an in silico method for identifying SC proteins in emerging model systems.

Building a visual consensus model of the SARS-CoV-2 life cycle
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A significant and positive outcome of the COVID-19 epidemic is the speed at which many members of the biological research community have been able to redirect and apply their specialized skills towards an increased understanding SARS-CoV-2. The rapid pace of publication and sharing of data, however, brings new concerns: what aspects of coronavirus biology are now well documented and understood, and what areas represent "black boxes" where there is inadequate research coverage or conflicting
results? How trustworthy are the currently available data, and how close are we to having a consensus model of different stages of the SARS-CoV-2 life cycle? We have recently launched a project that takes a novel approach for enabling rapid and transparent communication of the research community’s knowledge about the SARS-CoV-2 life cycle. We are developing a web-based tool that combines a detailed animated molecular working model of different stages of the coronavirus life cycle with mechanisms for the research community to annotate and discuss aspects of the model. Users will be able to step through the animated life cycle model and annotate specific molecular elements, both in space and time. The annotations from the research community, which will be visible publicly, will serve to show shifts in our understanding of the viral life cycle due to emerging research, as well help to develop updated visualizations -- including competing hypotheses -- that can be shared back with the research community. Ultimately, this project will result in a visualization of consensus model(s) of the coronavirus life cycle which can inform research communities as well as the public on our current understanding of the molecular mechanisms of SARS-CoV-2 viral infection.

Nuclear Shape and Size Control

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Chromatin organization regulates nuclear size in *Xenopus laevis* egg extract

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A fundamental question in cell biology is how size is regulated, from the organismal and tissue levels to cells and subcellular structures. Our lab studies nuclear size regulation using *Xenopus laevis* egg extracts, which provide a robust system to identify and characterize mechanisms of nuclear size regulation. Previously, we proposed that nucleoplasm (Npm2) drives nuclear growth by altering chromatin organization. To further investigate how chromatin organization might influence nuclear size, we studied nuclei assembled de novo in *X. laevis* egg extract. Nuclei treated with DNase or MNase were still import-competent but smaller than controls, suggesting that nuclear import is not sufficient to drive nuclear growth. Additionally, we used various small molecule inhibitors to alter chromatin organization. We found that increasing chromatin compaction leads to increased nuclear size while decreasing chromatin compaction resulted in smaller nuclei, suggesting that compacted chromatin can produce intranuclear force that promotes nuclear growth. We are currently testing this model using synthetic nuclei built around hydrogel beads that can be induced to expand. To test if large increases in DNA amount would affect nuclear size, we assembled nuclei using Axolotl sperm which contain 10-fold more DNA than *X. laevis* sperm. We observed a positive correlation between DNA amount and nuclear size. We next asked if nuclear F-actin might also contribute to nuclear size scaling. We assembled nuclei in actin-intact egg extract and then treated the nuclei with benzonase to degrade the DNA. These nuclei failed to grow, suggesting that F-actin cannot rescue nuclear growth in the absence of chromatin. We also found that the presence of dynamic F-actin in *X. laevis* egg extract affects chromatin structure and organization. In summary, we propose that chromatin-mediated intranuclear pushing forces on the nuclear envelope can drive nuclear growth, possibly by promoting lamin incorporation into the nuclear lamina.
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**The role of osmotic forces in nuclear size control**

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The size of the nucleus scales robustly with cell size so that the nuclear-to-cell volume ratio (N/C Ratio) is maintained during cell growth in many cell types. The mechanism responsible for this scaling is still mysterious. The N/C Ratio is not determined merely by DNA amount, but is influenced by factors such as properties of the nuclear envelope and nuclear transport. Here, we develop a physical-based model for nuclear size control based upon osmotic pressure. The nuclear envelope can be regarded as a semi-permeable barrier that allows water and small ions to pass, but is relatively impermeable to large macromolecules. These macromolecules generate colloid osmotic pressure differences between the compartments that inflate the nucleus. Nuclear size may arise as a passive outcome of the numbers of osmotically-active macromolecules in the nucleus and cytoplasm. To test this model, we analyze N/C Ratio regulation in fission yeast using tools such as microrheology with Genetically-Encoded Multimeric nanoparticles (GEMs) as a measure of macromolecular crowding. We find that the fission yeast nucleus, in contrast to the more complex mammalian one, behaves as an ideal osmometer, whose volume is determined in a linear relationship by its osmotic environment following the Van’t Hoff law. We investigate how the N/C Ratio is altered by nuclear export inhibition and application of external compressive forces as well as the effect of protein synthesis inhibition. These studies support a quantitative model that explains how nuclear size is determined by a physical mechanism that integrates molecular crowding, osmotic and compressive forces.

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**Filamin-2 organizes branched actin to facilitate nuclear migration through narrow constrictions**

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Nuclear migration through narrow constrictions is important for development, the maintenance of pro-inflammatory response, and implicated in metastatic cancers. While studies of migration through narrow spaces have been conducted *in vitro*, they have not been delineated *in vivo*. *Caenorhabditis elegans* P-cell nuclear migration through a small constriction serve as an *in vivo* model for this process. P-cells migrate from the lateral to the ventral part of the worm in the first larval stage and then develop into vulva and neuronal precursors. If P-cell nuclear migration fails, then the worm will not develop a vulva and some neurons, leading to Egl (egg-laying deficient) and Unc (uncoordinated) phenotypes. This process is typically mediated by the LINC complex, which spans the nuclear envelope and recruits dynein motors to the surface of nuclei. Mutations in LINC components, including the SUN protein UNC-84, lead to a temperature-sensitive P-cell nuclear migration phenotype. Since P-cell nuclei still migrate in the absence of LINC complexes, we hypothesized that a second, parallel, pathway contributes to P-cell nuclear migration. We performed a forward genetics *enhancer of the nuclear migration defect of unc-84 (emu)* screen and found three putative actin regulators: CGEF-1, TOCA-1, and FLN-2, indicating that actin plays a role in P-cell nuclear migration. Here we focus on the genetic characterization of *fln-2*. FLN-2 significantly enhanced the UNC-84 nuclear migration defect, and the CGEF-1; UNC-84 double mutant, but did not significantly modify the TOCA-1; UNC-84 double mutant. FLN-2 has many different isoforms,
and we were able to rescue the FLN-2;UNC-84 double mutant with a shorter isoform, devoid of the canonical filamin actin binding domain. We are in the process of GFP-tagging FLN-2 to observe its localization in vivo, as well as performing a domain analysis to determine how FLN-2 is contributing mechanically. We propose that nuclear deformation is facilitated by a branched actin network, which is organized by FLN-2.

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F-actin and Lamin A dependent regulation of nuclear shape in Xenopus egg extracts
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Xenopus egg extracts are a widely used system to study mechanisms of nuclear morphology regulation. The extracts are supplemented with calcium which promotes entry into interphase, sperm chromatin which acts a DNA source, and cycloheximide which blocks progression into mitosis. Traditionally, Xenopus extracts are prepared in the presence of the F-actin depolymerizing drug Cytochalasin B. However, recent studies have shown the importance of F-actin in nuclear morphology regulation, so we decided to prepare F-actin intact extract. Interestingly, while nuclei in Cytochalasin-treated extracts were mostly spherical, the nuclei formed in F-actin intact extracts were bilobed. Moreover, the bilobed nuclei had distinct characteristics, with the bigger lobe having higher actin intensity and fewer nuclear pore complexes, while lacking in Hoechst-staining DNA. We also observed a perinuclear actin ring-like structure within the nucleus using Lifeact-GFP and Phalloidin staining, suggesting that nuclear actin might contribute to the regulation of nuclear shape. In order to confirm this, we are planning to supplement the extract with exportin 6, which is responsible for nuclear export of actin. To further investigate the mechanism of actin-mediated nuclear morphology regulation, we tested various small molecule inhibitors of actin regulators. We found that bilobed nuclear morphology is dependent on formins, but not on myosin or Arp2/3. Upon inhibition of formins with SMIFH2, the nuclei were more round and the intranuclear actin intensity also decreased. Staining for various candidate formins revealed higher amounts of Diaph2 and actin in the bigger bleb lacking DNA. This suggests that the formin Diaph2 might be involved in intranuclear actin ring formation. Nuclear Lamin A/C is an intermediate filament protein known to regulate nuclear shape and is mutated in many laminopathies. Xenopus egg extracts lack Lamin A protein, so we hypothesized that the bilobed nuclear morphology seen in F-actin intact extract might be due to the absence of Lamin A. Interestingly, supplementing F-actin intact extract with recombinant Lamin A resulted in more round nuclei. This suggests that F-actin and Lamin A might counteract each other to regulate nuclear shape. We are currently testing this hypothesis in cultured mammalian cells.

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Macronuclear shape change in the giant ciliate, Stentor coeruleus

A fundamental question relating to cellular spatial patterning is how nuclei are shaped within cells. The giant ciliate Stentor coeruleus provides a unique opportunity to investigate the mechanisms behind nuclear shape, because Stentor takes this aspect of the nucleus to the extreme: Stentor possesses a macronucleus shaped like a string of spherical beads, totaling about 400 um in length. During cell
division, the macronucleus dramatically changes shape before dividing amitotically into the two daughter cells. The macronucleus condenses into a single sphere about 70 um wide, extends, and then remodulates. This entire process takes place in 2 hours near the end of cell division. We can experimentally induce this macronuclear shape change by causing Stentor to regenerate its oral apparatus. The morphological events during regeneration are similar to cell division, including the cycle of shape changes performed by the macronucleus. It is unclear how this extreme macronuclear shape change is regulated. What molecular and physical changes are driving macronuclear shape change during Stentor regeneration? Our first molecular foothold into this question comes from an RNAi screen of genes differentially expressed during regeneration in Stentor. We found that a homolog of the nuclear exportin gene CSE1 was among the top upregulated genes during this process. In other model systems, CSE1 is involved in nucleocytoplasmic transport by exporting importin-alpha from the nucleus. We tested the function of CSE1 in Stentor using RNAi. Knockdown of CSE1 results in Stentor with misshapen macronuclei that no longer are composed of circular nodes. Immunofluorescence of CSE1 in Stentor results in punctate staining in the cytoplasm with no staining in the macronucleus during the early stages of cell division and regeneration. When the macronucleus is condensed, CSE1 puncta appear in the interior of the macronucleus. Preliminary imaging data suggests that the macronuclear volume rapidly increases as the macronucleus is condensed, and decreases as the macronucleus elongates. Does nucleocytoplasmic transport via CSE1 help alter the volume of the macronucleus at this stage? Is this volume change necessary to set up the “beads on a string” morphology of Stentor’s macronucleus? We are currently investigating these questions.

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Exploring the dynamics of closed mitosis in fission yeast using Machine Learning

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Faithful cell division requires the spatiotemporal synchronization and coordination of multiple intracellular structural changes. While most of the genes involved in these events have now been identified, much remains to be discovered about the formation, localization and dynamics of components that make up the core division machinery. Many of these structures exhibit highly conserved and controlled spatiotemporal dynamics, but the mechanisms underlying them are not well understood. This work explores the capacity of machine learning (ML) to uncover novel mechanisms regulating the cell division machinery by analyzing large volumes of live imaging data. The fission yeast Schizosaccharomyces pombe accomplishes chromosome segregation while maintaining an intact nuclear envelope (NE) that needs to undergo a series of morphological changes. When projected in 2D, the
dividing nucleus appears as an elongating circle that eventually collapses into a dumbbell-like shape. This dumbbell further elongates until it splits into two daughter nuclei by local disassembly of its bridge midzone\(^1\). The pattern of distinct morphological transitions witnessed in this example of 'closed mitosis'\(^2\) is remarkably reproducible, making it particularly suitable for the investigation of control mechanisms. We first collected a large live imaging dataset of wild-type cells with a fluorescently tagged inner NE growing asynchronized in exponential log phase. We then developed a computational pipeline that batch processes the imaging data to isolate mitotic events and simultaneously extract shape measurements such as major axis and area from ML-generated 2D segmentations. Having obtained a multi-dimensional description of nuclei morphologies, we used a dimensionality reduction algorithm that projects our large dataset to a continuous 2D feature space while closely retaining its higher-dimensional structure. When examining the projection we can visualize global similarities and additionally identify patterns by tracking single-cell nuclear shape changes over time. By accurately characterizing morphological changes in this highly repeatable process, we can identify how these are altered upon biochemical disruption. This can help reveal the impact of specific processes and pathways on the biophysics of structures. Importantly, our findings show how ML approaches can be leveraged to extract a meaningful statistical description of biological processes. References: (1) Dey, G. et al. Closed mitosis requires local disassembly of the nuclear envelope. *Nature* 1-5 (2020). (2) Ungricht, R. & Kutay, U. Mechanisms and functions of nuclear envelope remodelling. *Nat Rev Mol Cell Biol* 18, 229-245 (2017).

**Control of nuclear shape changes in closed mitosis in fission yeast**

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In dividing eukaryotic cells, the nuclear envelope (NE) either disassembles before the chromosomes separate in an ‘open’ mitosis or divides into two after the chromosomes separate in a ‘closed’ mitosis. The NE remains intact in a closed mitosis, making it an excellent system to study the control of nuclear shape through the cell cycle. The fission yeast *Schizosaccharomyces pombe* undergoes closed mitosis. In early anaphase, the spherical nucleus elongates, but instead of forming a long uniform cylinder, the NE collapses in the middle to form a narrow bridge. This dumbbell shaped nucleus further elongates and the NE disassembles at the centre of the bridge to form two daughter nuclei (Dey et. al., Nature 2020). These shape changes in the nucleus have been reported in the literature but have not been studied quantitatively. We developed an analysis pipeline to identify dividing cells from spinning disk confocal time lapse movies of cells expressing a mNeonGreen-tagged inner NE marker, using an Ilastik algorithm. We used custom Fiji macros to extract various shape descriptors from the nuclei. We visualised the trajectories of these parameters over time using pandas and matplotlib libraries in Python. We hypothesise, as others have (Zhu et. al., Biophysical Journal 2016), that spindle elongation alone drives shape changes in the nucleus. To begin testing this idea we examined ‘solidity’ trajectories as a function of spindle length. Defined as the area of an object divided by its convex hull area, solidity serves as a reliable indicator of complex nuclear shape changes. We observed that the rate of change of solidity was
highest when the nucleus forms a dumbbell. These shape changes were observed reproducibly and consistently in over 1000 time lapse acquisitions collected across multiple independent experiments. We aim to inform a mechanical model of the NE with these data, incorporating the pushing forces of the spindle and the physical properties of the NE, such as tension and bending moments, to predict changes in the shape of the nucleus as the spindle elongates. References: Dey et al., Nature, doi: 10.1038/s41586-020-2648-3, Aug 2020. Zhu Q et al. Biophysical Journal, doi: 10.1016/j.bpj.2016.10.004, Nov 2016.

Signaling Receptors (RTKs and GPCRs)

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A ubiquitin based mechanism for the oligogenic inheritance of heterotaxy and heart defects

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Oligogenic inheritance and genetic modifiers are often invoked to explain human diseases in which monogenic defects, copy-number variants, or aneuploidy cannot explain the full burden of disease; however, the underlying biochemical and cell-biological mechanisms remain poorly understood. Here we describe an oligogenic mode of inheritance in the mouse involving the single pass transmembrane protein Megf8 and two partially redundant RING family E3 ubiquitin ligases, Mgrn1 and Rnf157. The inheritance of one pathogenic allele of either Megf8 or Mgrn1 results in normal development. However, inheritance of one pathogenic allele of both Megf8 and Mgrn1 produces a partially penetrant phenotype of heterotaxy with complex congenital heart defects (CHDs) that mimics the spectrum of CHDs seen in association with laterality defects in human birth registry data. Mechanistically, MEGF8 recruits MGRN1 to the plasma membrane to promote the ubiquitination of Smoothened (SMO), a key transducer in the Hedgehog (Hh) signaling pathway. Consequently, the abundance of SMO at the cell surface and primary cilium is reduced and sensitivity to Hh ligands is attenuated. Our work uncovers the molecular basis of a complex genetic inheritance mechanism and suggests that alterations in the strength of Hh signaling may be the causal factor responsible for the well-known association between heterotaxy and complex CHDs in humans.

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Proteomic analyses of EPH tyrosine kinase receptors reveal interactions with polarity proteins and a role in epithelial morphogenesis

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The Eph family of receptor tyrosine kinases (RTK) is the largest in humans. In contrast to other RTKs, Eph receptors (EphRs) cognate ligands, ephrins, are tethered to the cell surface. This results in EphRs-ephrin signaling being mainly involved in short-range cell-cell communication events that regulate cell adhesion, migration and tissue boundary formation. Although EphRs functions have been broadly studied, the molecular mechanisms by which they mediate these processes are far from being understood. To address this question, we sought to identify new downstream effector proteins for EphRs and to determine their requirement for EphR-regulated functions. To unravel EphR-associated signaling complexes under native conditions, we applied a mass spectrometry (MS)-based approach, namely BioID proximity labeling. We obtained a composite signaling network from EphA4, -B2, -B3 and -B4 receptors. This network comprises 395 proteins, most of which not previously linked to Eph signaling. We first examined the contribution of 14 candidates using a loss-of-function approach in an EphR-dependent cell sorting assay. We showed that depletion of a few candidates, including the signaling scaffold and polarity protein PAR3, blocks Eph-dependent cell sorting. Using affinity purification combined to MS, we further delineated a signalling complex involving C-SRC kinase (CSK), whose recruitment to PAR3 complexes is dependent on EphR signals. Furthermore, bioinformatics analyses revealed a strong enrichment for polarity proteins among those shared by the 4 EphRs that we explored, suggesting an involvement of EphRs in cell polarization. We showed that three of the fourteen EphRs (EphA1, -B2 and -B4) are expressed in Caco-2 epithelial cells when polarized. In addition, we found that EphA1 and EphB4 are localized at the basolateral domain within these cells. Interestingly, depletion of these two EphRs disrupted the morphogenesis of 3D spheroids. Our work suggests that a subgroup of EphRs is important for establishing morphogenesis of epithelial tissues. Overall, our work leads to a better understanding of the mechanisms by which EphRs signal at the membrane. It also gives insight into how Eph-mediated signaling pathways contribute to cell polarity.

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Serpentine Receptor 12 as a GPCR candidate in the parasitic protozoa Plasmodium falciparum

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Malaria causes millions of deaths worldwide and is considered a huge public health problem for underdeveloped countries. Plasmodium falciparum is the species that causes the most severe form of the disease, which causes death of around 500,000 people annually, causing clogging and rupture of blood vessels. GPCRs are a family of transmembrane proteins of immense biological importance, so that about half of the drugs on the market target some receptor in this family. GPCRs are found in abundance in multicellular eukaryotic organisms with the function of detecting molecules in the extracellular environment and transferring a stimulus into the cell. In parasitic protozoa, however, there is no description of a canonical GPCR. The characteristic signaling pathways and the presence of structurally similar proteins leads us to believe that these receptors are present in P. falciparum, but so far the existence of a member has not been proven. To test the hypothesis of GPCR existence in P. falciparum we used a series of Bioluminescence and Bioluminescence Resonance Energy Transfer (BRET)-based biosensors to investigate the signaling activity of P. falciparum Serpentine Receptor 12
(PfSR12) in a mammalian cell system. Using an Obelin based biosensor, thrombin promoted a PfSR12-dependent cytosolic Ca²⁺ rise in HEK293 cells. This Ca²⁺ mobilization was accompanied by DAG formation and PKC activation as detected using DAG and PKC BRET-based biosensors indicating a Gqq coupled PLC/IP3 signaling pathway. The role of Gq was confirmed using Gq/11 knockout HEK293 cells as well as the Gq-selective inhibitor, YM254890. Further investigation revealed that PfSR12 is not itself a thrombin receptor but rather promotes the increase of cell surface expression of an endogenous thrombin receptor. This chaperone-like effect was not selective for thrombin receptors as PfSR12 expression also promoted an increased muscarinic type 3 receptor (M3R)-promoted DAG and PKC responses. This increase response was accompanied by an increase in surface expression of M3R. Our data indicate that PfSR12 have strong GPCR structural and functional characteristics, acting as a chaperone and increasing the expression of several GPCRs resulting in augmented responsiveness to various hormones of mammalian cells that could contribute to the deleterious effects of *Plasmodium falciparum* infection.

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**Casein kinases regulate the stability of the glucose sensing receptors Rgt2 and Snf3 in yeast**

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Casein kinases regulate the stability of the glucose sensing receptors Rgt2 and Snf3 in yeast. Emma Mohler*, Sarah Melton, Dajeong Jung and Jeong-Ho KimThe yeast Saccharomyces cerevisiae senses extracellular glucose through the two glucose-sensing receptors Rgt2 and Snf3. This process requires the plasma membrane-tethered casein kinases Yck1 and Yck2 (yeast casein kinases (Ycks), the homologs of the casein kinase 1-gamma (CK1γ), but their exact role is not clear. Here, we provide evidence that the Ycks act upstream of the glucose receptors in glucose sensing. Rgt2 is stable at the plasma membrane in the presence of glucose, but it is endocytosed and targeted to the vacuole for degradation when glucose is depleted from the medium. However, Rgt2 is constitutively degraded in the absence of the Ycks, suggesting that the kinases act to protect the glucose receptors from degradation. The Akr1 palmitoyl transferase tethers Yck1 to the plasma membrane, and thus the Ycks are diffused throughout the entire cell. Interestingly, Rgt2 is unstable (degraded) in the akr1α strain even under high glucose concentrations, reinforcing the view that the Ycks play a key role in regulating the stability of the glucose receptors. The glucose receptors have long C-terminal, presumably cytoplasmic, tails, which are believed to constitute the signaling domain of the receptor proteins. The Ycks phosphorylate the C-terminal domain (CTD) of Rgt2 in a glucose-dependent manner. Indeed, the Rgt2-CTD interacts with the Ycks in a yeast two-hybrid assay. These results provide evidence that, in response to glucose, the Ycks phosphorylate the glucose receptors to prevent them from degradation and reveal new insights into the receptor(Rgt2/Snf3)-mediated glucose sensing.

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**Spatial and Temporal Regulation of Sensors of a Mitogen Activated Protein Kinase Pathway Contribute to a Pathway-Specific Response**

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MAPK pathways are evolutionarily conserved signaling modules that regulate cell differentiation and the response to stress. In most organisms, MAPK pathways are regulated by proteins that can function in multiple pathways. Despite sharing components, different MAPK pathways sense specific stimuli and generate a unique response. The budding yeast *Saccharomyces cerevisiae* contains multiple MAPK pathways that share components. One allows cells to undergo filamentous growth in response to nutrient availability (fMAPK), and the other allows cells to respond to changes in external osmolarity (HOG). The pathways are regulated by different mucin-type sensors that converge on a common protein module that contains the tetraspan protein, Sho1p; Rho GTPase, Cdc42p; and the MAPKKK, Ste11p. We previously showed that the activity of the fMAPK pathway is cell-cycle regulated and peaks at M/G1. By comparison, the HOG pathway did not show cell-cycle regulation. The different regulatory patterns of these MAPK pathways could be explained by differences in the levels of the signaling mucins that regulate these pathways. The abundance of the mucin Msb2p (fMAPK) increased at M/G1. By comparison, the levels of the mucin Hkr1p (HOG), did not change throughout the cell cycle. We also found that the tetraspan protein, Sho1p, which interacts with both mucins, showed a dynamic localization pattern throughout the cell cycle and localized to the mother-bud neck during M/G1. Using indirect immunofluorescence to measure active Kss1p, the MAP kinase of the fMAPK pathway, we found that Sho1p localization at the mother-bud neck correlated with increased fMAPK activity. The importance of Sho1p localization at the mother-bud neck for fMAPK function was further corroborated in a conditional septin mutant, *cdc12‐6*, which had mislocalized Sho1p and low fMAPK activity compared to the wild type. Moreover, a version of Sho1p that was uniformly targeted to the plasma membrane through N-terminal myristoylation, prevented Sho1p from enriching at the mother-bud neck and showed a reduction in fMAPK pathway activity. Together, the study identifies two ways MAPK pathways exhibit selectivity at the sensor level: one by altering the levels of a mucin sensor, and the other by regulating the localization of its direct effector. Spatial and temporal regulation of MAPK pathways may direct their functions in specific contexts.

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**Glucose regulation of cell surface levels of the glucose sensing receptors Rgt2 and Snf3 in yeast**

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Glucose regulation of cell surface levels of the glucose sensing receptors Rgt2 and Snf3 in yeast*Rebeca Rodriguez* and Jeong-Ho Kim*Most organisms sense glucose and utilize it efficiently, but its underlying mechanisms are not well understood. In the yeast *Saccharomyces cerevisiae*, the two plasma membrane proteins Rgt2 and Snf3, known as glucose sensors/receptors, sense extracellular glucose levels. Rgt2 appears to sense high glucose levels and Snf3, low glucose levels, respectively, perhaps due to their different affinities for glucose. Here, we provide evidence that cell surface levels of the glucose sensing receptors is differentially regulated in response to changes in glucose concentration. Both Rgt2 and Snf3 are endocytosed and degraded in the vacuole in the Rsp5 ubiquitin ligase-dependent manner when glucose is removed from the growth medium. Constitutively active receptors are not degraded, whereas signaling defective receptors are constitutively degraded. While glucose starvation-induced endocytic degradation of Rgt2 and Snf3 receptors provides a mechanism for downregulating their cell surface expression, *SNF3* gene expression is also repressed by high glucose. Thus, Rgt2 is the major glucose receptor in cells grown on high glucose, whereas Snf3, in cells grown on low glucose, enabling yeast cells to respond quickly to glucose over a wide range of concentrations. When expressed
from a glucose-independent promoter, however, Rgt2 and Snf3 display similar expression patterns and their functions are interchangeable and indistinguishable. Together, our results provide evidence that the different functions of Rgt2 and Snf3 in glucose sensing are likely due to differences in their expression patterns, rather than due to the results of the two glucose sensing receptors exhibiting different functions depending on their affinities for glucose.

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**Regulation of α-arrestin function in GPCR signaling and trafficking**

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The ubiquitously expressed β-arrestins are known to play key roles in regulating G protein-coupled receptor (GPCR) desensitization, internalization, and signaling. Humans also express a newly discovered family of arrestins, termed α-arrestins, whose function remains poorly understood. While β- and α-arrestins share minimal sequence homology, they are predicted to be structurally similar, except that β-arrestins contain clathrin binding motifs within their C-tails, which are known to be important in facilitating receptor internalization, and α-arrestins contain PPxY motifs in their C-tails, which are known to recruit WW-domain containing proteins, such as Nedd4-family E3 ubiquitin ligases. This suggests a separate role for α-arrestins, although they may be subject to similar regulatory mechanisms, including phosphorylation, ubiquitination, and conformational change. Additionally, yeast, which do not express β-arrestins, express arrestin-related trafficking adaptors (ARTs), which are predicted to be structurally similar to human arrestins and contain PPxY motifs. ARTs have been well-characterized as key regulators of receptor trafficking in yeast and are also regulated through post-translational modification. Recently, we demonstrated a role for arrestin domain-containing protein-3 (ARRDC3), a mammalian α-arrestin, in regulating trafficking of protease-activated receptor-1, a GPCR for thrombin. However, while GPCR regulation of β-arrestin function has been well characterized, it remains entirely unknown how GPCRs regulate α-arrestins, such as ARRDC3, or how such regulatory mechanisms might impact α-arrestin function. Here, we examine how PAR1 signaling regulates ARRDC3 function in facilitating PAR1 trafficking. Using a variety of biochemical and microscopy-based techniques, we show that PAR1 may regulate ARRDC3 through modulation of post-translational modifications, and that identified ARRDC3 regulatory domains may play a role in regulating ARRDC3 function through controlling its subcellular localization and recruitment of interaction partners. We additionally will use a variety of approaches to elucidate the role of ARRDC3 regulatory mechanisms in PAR1 trafficking and signaling. Interestingly, ARRDC3 has been identified to be a tumor suppressor in invasive breast cancer, functioning at least partially through its role in controlling PAR1 signaling and trafficking. Therefore, the results of these studies will not only advance our understating of how α-arrestins are regulated but may also lead to the identification of novel therapeutic targets to prevent or treat invasive breast cancer.

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**Optogenetic control of the Sphingosine-1-phosphate receptor 1 enables light-mediated regulation of endothelial permeability and angiogenesis**

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Sphingosine-1-phosphate receptor 1 (S1PR1) is a critical regulator of endothelial cell (EC) migration, cytoskeletal structure, capillary-network formation, sprouting angiogenesis, and vascular maturation. Here we developed a strategy for controlling endothelial barrier function and sprouting angiogenesis using an engineered photoactivatable (PA)-S1PR1 receptor. Regulation of signaling and cell function by PA-S1PR1 was demonstrated in human pulmonary arterial endothelial cells (HPAECs). Following illumination with bluelight, PA-S1PR1 induced activation of known downstream signaling pathways (AKT, Erk1/2). The kinetic and the extent of activation correlated with what was observed in HPAECs treated with 1µM S1P. Furthermore, activation of PA-S1PR1 led to enhancement of endothelial barrier function and stimulation of sprouting angiogenesis in vitro. Thus, these findings suggest that engineered PA-S1PR1 can be used to mimic its function in living cells and dissect S1P signaling with precise temporal and spatial resolution.

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R-spondins engage heparan sulfate proteoglycans to potentiate WNT signaling

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R-spondins (RSPOs) amplify WNT signaling during development and regenerative responses. We previously demonstrated that RSPOs 2 and 3 potentiate WNT/beta-catenin signaling in cells lacking leucine-rich repeat-containing G-protein coupled receptors (LGRs) 4, 5 and 6. We now show that heparan sulfate proteoglycans (HSPGs) act as alternative co-receptors for RSPO3 using a combination of ligand mutagenesis and ligand engineering. Mutations in RSPO3 residues predicted to contact HSPGs impair its signaling capacity. Conversely, the HSPG-binding domains of RSPO3 can be entirely replaced with an antibody that recognizes heparan sulfate (HS) chains attached to multiple HSPGs without diminishing WNT-potentiating activity in cultured cells and intestinal organoids. A genome-wide screen for mediators of RSPO3 signaling in cells lacking LGRs 4, 5 and 6 failed to reveal other receptors. We conclude that HSPGs are RSPO co-receptors that potentiate WNT signaling in the presence and absence of LGRs.

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Analysis of the specificity of adaptor proteins NCK1 and NCK2

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Signaling pathways downstream of receptor tyrosine kinases (RTK) are essential for organogenesis and homeostasis. RTK signals are often relayed via adaptor proteins that recruit target proteins to form receptor-specific signaling networks. Adaptor proteins NCK1 and NCK2 are composed of a single SH2 (Src Homology 2) interaction domain that binds phosphorylated tyrosine containing motifs, and three SH3 domains binding proteins containing poly-proline motifs. Although these proteins are often considered redundant, we have shown that each specifically associates with a subset of targets through the identification of their interaction networks by proteomic approaches such as AP-MS and BioID. However, the molecular mechanisms allowing this specificity in their interactions are still unresolved, given the strong homology between the two proteins. Among NCK2 specific interactors, we focused on PKP4 (plakophilin-4), a protein involved in cell adhesion and actin cytoskeleton organization. We confirmed that the PKP4-NCK2 interaction is specific and dependent on the SH2 domain of NCK2. However, the SH2 domain alone does not recapitulate the binding specificity to NCK2. We hypothesized that intrinsically disordered regions between the conserved SH2-SH3 domains (i.e. interdomain) of NCK1 and NCK2 proteins dictate their specificity. Using AP-MS, we compared the interactome wild-type and NCK1/2 chimeras (NCK1*/NCK2*), in which only SH2/3 domains were inverted from the backbone (e.g. NCK1* bears NCK1 SH2/3 domains and NCK2 interdomain regions). We observed both losses and gains of interaction partners between the WT and chimeric proteins. Our data suggest that its specificity for NCK2 SH2 domain is regulated by interdomains regions, as PKP4 associates with NCK2, NCK1* and NCK2*. Our work will explain how adaptor proteins fine-tune signals downstream of RTKs, and how different RTKs can use the same signaling effectors to transmit signals yet establish distinct phenotypes.

P1024

Regulation of Epidermal Growth Factor Receptor by Clathrin-Associated Kinases

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The Epidermal Growth Factor (EGF) Receptor (EGFR) is a receptor tyrosine kinase (RTK) that controls many key components of cell physiology, including proliferation, survival, and metabolism. When upregulated, EGFR drives tumor growth and progression in several types of cancer. EGF stimulation elicits EGFR phosphorylation and activation of phosphatidylinositol-3-kinase (PI3K), leading to Akt activation by its phosphorylation on T308 and S473. EGF-stimulated Akt phosphorylation is dependent on clathrin coated pits (CCPs) at the plasma membrane, but not receptor endocytosis. CCPs are 50-100 nm protein assemblies that are well-known endocytic portals and lead to eventual receptor downregulation. Importantly, we previously uncovered that CCPs are also required for Akt activation, likely by acting as protein and lipid scaffolds to coordinate signaling intermediates. One of the many proteins that are known to bind to clathrin directly is Activated Cdc42 Kinase 1 (Ack1), a non-receptor tyrosine kinase implicated in oncogenic RTK signaling and tumor cell survival. Ack1 also interacts with EGFR and directly phosphorylates Akt at Y176, a regulatory site distinct from those required for canonical Akt activation. In addition to clathrin, Ack1 also interacts with a multitude of other proteins. How Ack1 regulates the PI3K/Akt signaling pathway, and how binding of Ack1 to clathrin controls this phenomenon, remains poorly defined. Here, we examine how Ack1 contributes to EGFR signaling. Using siRNA silencing of Ack1 and/or the related isoform Ack2, alone or in combination with inducible
expression of fluorescent or mutant Ack constructs, we examined the role of Ack1 in EGFR signaling. Moreover, using various microscopy and image analysis techniques, we examined the mechanism of recruitment of Ack1 to CCPs. Our results indicate a critical role for Ack1 in EGFR signaling, in particular for the activation of PI3K-Akt signaling. The improved understanding of the regulation and outcome of EGFR signals by proteins such as Ack1 and scaffolds such as clathrin-coated pits, can lead to the development of novel cancer treatments.

Spindle Assembly Checkpoint

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A tripartite mechanism catalyzes formation of the Mad2-Cdc20 complex at unattached kinetochores to activate the spindle assembly checkpoint

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During mitosis, the spindle assembly checkpoint protects against aneuploidy by monitoring the interaction between kinetochores and spindle microtubules. When devoid of bound microtubules, kinetochores recruit checkpoint proteins to generate a diffusible inhibitor, known as the mitotic checkpoint complex, that prevents mitotic exit. Specifically, unattached kinetochores catalyze a rate-limiting reaction in the formation of the mitotic checkpoint complex: the association of Cdc20 with Mad2. Despite evidence in support of this model, it is unclear why formation of Mad2-Cdc20 complexes is kinetically limiting and how unattached kinetochores specifically overcome this kinetic barrier. A major challenge to studying checkpoint activation in living cells is the presence of two functionally distinct pools of Mad2 at the kinetochore: a stable Mad1-bound scaffold pool (Mad1-Mad2) and a cycling pool that fluxes through Mad1-Mad2 and complexes with Cdc20 to generate the diffusible mitotic checkpoint complex. Here, using C. elegans embryos, we describe a fluorescent probe that specifically detects interaction of the cycling pool of Mad2 with Cdc20 at unattached kinetochores. Using this probe, we investigated the mechanism of kinetochore-catalyzed Mad2-Cdc20 assembly. Our data show that catalysis depends on three key events at unattached kinetochores: 1) the recruitment of Cdc20 to unattached kinetochores via the kinetochore scaffold Bub1; 2) the interaction between Mad1-Mad2 and a conserved motif in Bub1 that geometrically constrains the positions of Mad1-Mad2 and Cdc20; and 3) the phospho-dependent binding of the Cdc20 N-terminus to the C-terminal RWD domain of Mad1 that primes Cdc20 for interaction with Mad2. Thus, unattached kinetochores act in a tripartite manner to locally overcome the intrinsic kinetic barrier to Mad2-Cdc20 interaction, with Bub1 acting as a “matchmaker” that facilitates the interaction between Cdc20 and Mad2. Such a mechanism explains selective activation of the checkpoint at unattached kinetochores and not in the cytosol, a critical event responsible for ensuring genomic integrity during cell division.
Role of Microtubule Generated Tension in Accurate Chromosome Segregation

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Improper chromosome segregation during cell division can be deleterious by causing birth defects and aneuploidy, or even resulting in the development of tumors. Chromosome biorientation is crucial to ensure faithful segregation, wherein kinetochore-microtubule attachment as well as microtubule-generated tension across the attachments of sister chromatids is required to satisfy the Spindle Activation Checkpoint (SAC) and progress into anaphase. It is therefore, essential to understand the pathways leading to activation of the Spindle Checkpoint and associated mechanisms that promote chromosome biorientation prior to anaphase. Toward this goal, we developed a novel Taxol-sensitive yeast strain with which we isolated the tension-specific and attachment-mediated responses at kinetochores, which both induce delay in anaphase onset. This revealed discrete functions for SAC proteins; whereas Bub1, Bub3, Mad1, Mad2 and Mad3 are all required for SAC signaling due to unattached kinetochores, Bub1 and Bub3 specifically facilitate a transient delay in response to low tension at attached kinetochores. When microtubule-kinetochore attachments lack sufficient tension, Aurora B kinase (Ipl1 in yeast) phosphorylates kinetochore-associated proteins to promote detachment and subsequent Spindle Checkpoint activation. Aurora B also functions to prevent anaphase onset independent of producing unattached kinetochores, but the mechanistic details remain obscure. Our Taxol-sensitive yeast model offers a unique opportunity to investigate the functions of microtubule-generated tension and associated proteins, such as Aurora B, in preventing anaphase onset, independent of their roles in generating unattached kinetochores. We are currently studying the mechanisms through which Ipl1 phosphorylates kinetochore-associated proteins, and whether it functions in the tension-mediated delay. Our overall goal is to understand the inclusive impact of the tension- and attachment-based responses in mediating the overall delay and ensuring chromosome biorientation prior to anaphase.

Cell, spindle, and pole sizes along with microtubule density control mitotic duration by affecting the timing of checkpoint silencing

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Mitosis proceeds through a defined series of events that is largely conserved, but the total length for their completion can vary in different cells and organisms. In many systems, mitotic duration depends on the time required to satisfy and silence the spindle assembly checkpoint (SAC), the surveillance mechanism that monitors kinetochore-microtubule attachments and halts mitosis until all kinetochores are bound to spindle microtubules. Because SAC silencing involves trafficking SAC molecules among kinetochores, spindle, and cytoplasm, the size and geometry of the spindle relative to cell volume are expected to affect mitotic duration by influencing the timing of SAC silencing. However, a thorough understanding of whether and how the time required for SAC silencing depends on cell size and spindle dimensions is lacking. Here, we use tetraploidy, a system previously found to have disparate effects on cell and spindle size, to study the relationship between cell size, mitotic spindle geometry, and SAC function. Tetraploid (4N) clones derived from DLD-1 colorectal cancer cells displayed varying degrees of
cell and spindle size scaling. We found that some 4N clones increased cell/nuclear volume nearly two-fold, consistent with the increase in DNA content, while others increased to a lesser extent compared to the parental diploid cells. Even among similarly sized 4N clones, spindle length and volume differed, but the small 4N clones consistently formed taller spindles and rounder mitotic cells than the large 4N clones. Moreover, the small 4N clones had longer mitotic durations than the DLD-1 cells, which was due to differences in metaphase duration. Leveraging a previous mathematical model for spatiotemporal regulation of SAC silencing, we show that the difference in metaphase duration, i.e. SAC silencing time, can be explained by the distinct spindle microtubule densities and sizes of the cell, spindle, and spindle poles in the 4N clones. Lastly, in a small 4N clone characterized by a unique spindle shape and the longest metaphase duration, we demonstrate that altering spindle geometry reduced this delay, consistent with a prediction of the mathematical model. Our results suggest that spindle size does not always scale with cell size in mammalian cells, and tetraploidy can affect several aspects of mitosis, including spindle shape, cell rounding, mitotic duration, and SAC silencing kinetics, which may have relevance for its role in cancer.

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**Dynein-mediated eviction of checkpoint effectors from kinetochores does not require dynein motility along microtubules**

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Kinetochore-microtubule attachment status is monitored by the spindle assembly checkpoint, a molecular surveillance system that generates a “wait anaphase” signal at unattached kinetochores. During mitosis in metazoan, the minus end-directed motor protein cytoplasmic dynein-1 localizes to kinetochores at high levels in early mitosis, and to progressively lower levels as mitosis proceeds. Its recruitment is thought to primarily depend on the Spindly-RZZ (Rod-Zwilch-ZW10) complex, which is targeted to kinetochores in a poorly understood manner that likely relies on Mps1-mediated phosphorylation of “MELT” repeats in the large kinetochore scaffolding protein KNL1. Upon stable microtubule attachment, kinetochore-localized dynein has been proposed to actively transport spindle checkpoint proteins away from the kinetochore and toward the spindle poles using its microtubule-dependent motor activity, thus silencing the checkpoint and allowing for anaphase progression. Although it well established that dynein is required for silencing of the spindle checkpoint, such motility-driven eviction of checkpoint proteins by dynein along spindle microtubules has not been directly observed in mammalian cells. Moreover, the molecular signal that initiates dynein eviction from its kinetochore binding site upon microtubule attachment remains unknown. Using an endogenously-tagged dynein cell line (GFP-DYNC1H1, encoding the dynein heavy chain), we are now able to directly interrogate native dynein localization at kinetochores, and determine the requirements for its eviction therefrom. Although GFP-dynein puncta exhibit dynamic localization during mitosis, we observed no clear evidence of dynein poleward transport along spindle microtubules. Using these cells in combination with the mitotic and microtubule poisons reversine (to inhibit Mps1 activity) and nocodazole (to depolymerize microtubules), we developed a “kinetochore eviction” assay to investigate how dynein eviction is coupled to spindle checkpoint protein removal, and the role of kinetochore-microtubule attachments in this process. As expected, we find that Mad2 (but not BubR1) requires dynein for eviction from kinetochores. Surprisingly, the complete eviction of Mad2 - and dynein to a
lesser extent - from kinetochores could be initiated by the addition of reversine, but did not require the presence of microtubules. These data suggest that the role of dynein in spindle checkpoint protein eviction is not to transport Mad2 and other spindle checkpoint effectors along microtubules. Rather, our data support a model in which dynein-mediated eviction of checkpoint effectors relies on the elimination of its Mps1-dependent kinetochore binding site.

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Investigating the mechanism of BUB-1/BUB-3 kinetochore recruitment in the C. elegans one cell embryo

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The Bub1/Bub3 complex is central to spindle checkpoint signaling and accurate chromosome segregation during mitosis. The current model for Bub1/Bub3 kinetochore localization, based on structural and biochemical studies, is that phosphorylation of “MELT” repeats in the N-terminus of the Knl1 scaffold is recognized by the WD40 domain of Bub3, in conjunction with a loop from Bub3-bound Bub1 (Primorac et al, 2013, eLife 2:e01030). Here, we investigate whether this mechanism accounts for BUB-1/BUB-3 complex localization to kinetochores in the one-cell C. elegans embryo. In C. elegans, BUB-1 depletion is embryonic lethal while BUB-3 depletion or genetic deletion is viable, suggesting the presence of BUB-3-independent pools of BUB-1 at the kinetochore. Consistent with this idea, selectively mutating the recognition of phosphorylated KNL-1 MELT repeats by BUB-3 reduced but did not eliminate kinetochore recruitment of BUB-1/BUB-3. Inhibiting phosphorecognition of MELTs by BUB-3 did, however, impair spindle checkpoint signaling. The kinase that phosphorylates KNL-1 MELT repeats is PLK-1 in C. elegans; in human cells, PLK-1 has been shown to bind to BUB-1 via a Cdk-primed motif. Mutating the PLK-1 interaction motif on BUB-1 reduced BUB-1 kinetochore accumulation, suggesting that BUB-1 promotes its own phospho-dependent accumulation in a positive feedback loop. In contrast to mutation of phosphorecognition by the BUB-3 WD40 domain, deletion of the TPR domain at the N-terminus of BUB-1 eliminated kinetochore localization of BUB-1; in addition, the BUB-1 TPR domain by itself, which lacks the binding site for BUB-3, localized to kinetochores in the absence of endogenous BUB-1. In human cells, hydrophobic KI motifs on Knl1 are known to directly bind to the Bub1 TPR, suggesting that a similar interaction may contribute to BUB-1/BUB-3 kinetochore localization in C. elegans; however, the KI motifs do not appear to be conserved beyond vertebrates and we are currently attempting to identify potential direct links between the BUB-1 TPR and resident kinetochore components such as KNL-1. Our results suggest that BUB-1 kinetochore recruitment requires the BUB-1 TPR domain, which brings a pool of BUB-1-bound PLK-1 to phosphorylate MELT repeats on KNL-1 and drive a positive feedback loop elevating BUB-1/BUB-3 kinetochore localization. Our data suggest that this positive feedback loop enables BUB-1 to promote APC/C activation, supporting the model that BUB-1 has the ability to accelerate cell cycle progression, in addition to its well-known role in delaying progression through the spindle checkpoint. We suggest that the activity of BUB-1 is context-dependent, with microtubule attachment controlling its ability to accelerate versus delay anaphase onset.
A phosphoproteomic approach to understanding the role of the Bub1 kinase domain in chromosome segregation

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Accurate chromosome segregation is crucial for cell survival and preventing aneuploidy. To achieve this, a bioriented spindle must be formed where each kinetochore is attached to a microtubule emanating from the opposite spindle pole body. These bioriented attachments are reinforced by microtubule-generated tension at kinetochores to ensure proper spindle assembly. Both microtubule attachments and tension at kinetochores are vital for the formation of a bioriented spindle needed for proper chromosome segregation. Bub1 and Bub3 are key proteins in the Spindle Assembly Checkpoint (SAC), a signaling cascade that prevents anaphase onset if one or more kinetochores are unattached. Bub1 and Bub3 are recruited to unattached kinetochores to catalyze the formation of the Mitotic Checkpoint Complex (MCC) which prevents anaphase by sequestering Cdc20. Formation of the MCC leads to a delay in anaphase onset by preventing activation of the APC/C (anaphase promoting complex), resulting in metaphase arrest until every kinetochore attaches to a microtubule. Recently, Bub1 and Bub3 have been shown to delay anaphase onset in the presence of attached, low tension kinetochores independent of the canonical SAC. The Bub1 kinase domain is not needed for SAC signaling, but is needed for localization of Sgo1, a protein implicated in tension sensing and localization of error correction machinery. How specifically the Bub1 kinase domain responds to tensionless kinetochores and promotes chromosome segregation remains unclear. To address this, we used global quantitative mass spectrometry in budding yeast cells to investigate differences in phosphorylations between cells with and without the Bub1 kinase domain. Before protein extraction, cells were treated with nocodazole to disrupt attachments and tension at kinetochores, isolating tension-specific phosphorylations of the Bub1 kinase domain. We identified over 15,000 phosphorylations, and of the total down regulated phosphorylations in the Bub1-Δkinase cells, about 350 sites had a significant p-value (≤ 0.005) and enrichment (log₂ fold change). These significantly down regulated phosphorylations had a GO (Gene Ontology) term category enriched for mitotic cell cycle process (GO:1903047) and some were found on proteins implicated in anaphase onset timing and cohesion maintenance and establishment. These results suggest the importance understanding how the Bub1 kinase domain ensures chromosome segregation through regulation of anaphase onset timing and sister chromatid cohesion.

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Fin1-pp1 promotes kinetochore removal of spindle assembly checkpoint proteins by dephosphorylating a kinetochore protein ndc80

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The spindle assembly checkpoint (SAC) prevents anaphase onset in response to chromosome attachment defects, and SAC silencing is essential for anaphase onset. Following anaphase onset, phosphatase activity increases to promote turnover of kinetochore-microtubule attachments. Activated Cdc14 phosphatase dephosphorylates CDK substrates to facilitate anaphase progression and mitotic exit. In budding yeast, Cdc14 dephosphorylates Fin1, a regulatory subunit of protein phosphatase 1 (PP1), to enable kinetochore localization of Fin1-PP1. We previously showed that kinetochore-localized
Fin1-PP1 promotes the removal of the SAC protein Bub1 from the kinetochore. We report here that Fin1-PP1 also promotes the removal of the SAC protein Bub3, the partner of Bub1. Moreover, the kinetochore localization of Bub1/Bub3 during anaphase is dependent on Aurora B/Ipl1 kinase activity. We further show that Fin1-PP1 facilitates dephosphorylation of the kinetochore protein Ndc80, a known Ipl1 substrate. This dephosphorylation reduces kinetochore association of SAC proteins Bub1/Bub3 during anaphase. In addition, we found that untimely Ndc80 dephosphorylation compromises SAC activity and causes viability loss in response to tensionless attachments. These results suggest that the precise localization of Fin1-PP1 to the kinetochore is crucial to ensure the functional window for the SAC and therefore faithful chromosome segregation.

Investigating the mechanism by which cell size impacts spindle assembly checkpoint strength in Caenorhabditis elegans
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The spindle assembly checkpoint (SAC) monitors for spindle attachment defects in order to ensure accurate chromosomal segregation. Although the core mechanism is highly conserved, there exists variation between organisms and cell types in the duration of mitotic arrest following SAC activation, hereafter referred to as SAC strength. Previous studies have shown that cell size plays a role in the strength of the SAC response, with smaller cells exhibiting a stronger SAC. Two hypotheses have been proposed to explain why SAC strength can scale with cell size: (1) an increase in the ratio of kinetochore to cytoplasm favors synthesis of the mitotic checkpoint complex (MCC), the primary SAC effector, in small cells; and (2) an increase in the ratio of nucleoplasm to cytoplasm provides smaller cells with a higher concentration of pre-mitotic MCC, and thus a stronger SAC upon mitotic entry. In C. elegans, the first hypothesis may explain the increase in SAC strength with decreasing cell size during early embryonic cleavage divisions; whether the second also plays a role has not been addressed. We have shown previously that experimental manipulation of embryo size has a greater impact on SAC strength than similar changes in cell size that occur during cleavage divisions. Here, we assess whether, in altering embryo size, we are inadvertently changing concentration of SAC proteins and thus SAC strength. This would occur if SAC proteins are pre-loaded into the oocyte nucleus, which is formed prior to the events that set the final oocyte, and thus embryo, size. To test this, we combined RNAi-mediated knockdowns to generate large and small oocytes and embryos, with quantification of fluorescence intensities for an endogenously GFP-tagged version of the core SAC protein MDF-1/Mad1. We found that small oocytes appear to have a higher nucleoplasm-to-cytoplasm ratio, at least with respect to MDF-1/Mad1. We predict that this should translate into a higher concentration of MDF-1/Mad1 in the resulting small embryos and are currently performing experiments to test this. These results suggest that scaling of SAC strength with embryo size in C. elegans is influenced both by the ratio of cytoplasm to kinetochore and by the concentration of SAC proteins.
Mitotic DNA damage induces chromosomal congression defects, SAC arrest and micronuclei formation

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Little is known regarding the impact of DNA damage occurring in mitosis. Here, we set out to investigate the impact of DNA damage in mitotic human cells. Using a cell cycle/DNA damage small-molecule library we tested 450 compounds and selected 14 DNA-damaging agents that induced different types of DNA lesions and are used in the clinic (e.g. Mitomycin C, Carboplatin, Actinomycin C). Combining single-cell back-tracking with γH2AX levels we started by ensuring that the damage occurred specifically in mitosis. Based on live-cell imaging we show that, regardless of the compound, the mitotic damage induces severe chromosome congression defects and prolonged mitotic arrest. This delay is Spindle Assembly Checkpoint (SAC)-dependent as we observed misaligned chromosomes at the poles with Mad1-positive kinetochores (KTs). The majority of these chromosomes never align, and these cells resume mitosis with formation of micronuclei. Hence, mitotic DNA damage interferes with chromosome congression, therefore with SAC silencing. However, mitotic DNA damage does not directly activate SAC since in metaphase arrested cells inflicted DNA damage does not trigger the recruitment of Mad1 and therefore not reactivating SAC signaling. Furthermore, we also show that mitotic DNA damage does not contribute to the mitotic arrest, as SAC-inhibited cells, with or without damage, spend the same time in mitosis. Additionally, MG132 treatment reverts mitotic timings and cell fate in the same extension. Importantly, we show that SAC is the only mechanism that provides time to the mitotic cell and mitotic DNA damage induces a mitotic delay through unattached KT's. Critically, we found that in nocodazole-arrest cells SAC is impaired only with DNA-damaging agents that act through DNA intercalation (Actinomycin D and Doxorubicin). Mechanistically, we found that these compounds, by interfering with centromeric structure, compromise Aurora B localization and Mad1 recruitment. Overall, our data show that mitotic DNA damage induces chromosomal congression defects that culminate in the formation of micronuclei. We are currently investigating how mitotic DNA damage induces chromosomal congression defects. Curiously, we found that CENP-E inhibition phenocopied the mitotic DNA damage effect. Our findings bring a new insight to understand heterogeneity and drug resistance in tumors treated with DNA-damaging agents.
Synaptic Cytoskeletons

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Selective spine pruning through IgCAMs and Semaphorins is achieved through actin cytoskeletal signaling in cortical pyramidal neurons

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Dendritic spines of cortical pyramidal neurons are initially overproduced and subsequently eliminated in the adolescent brain to achieve an appropriate excitatory balance in mature circuits by obscure mechanisms. We have identified a novel combinatorial mechanism of selective spine pruning in which class 3 Semaphorins (Sema3) and Ig-class adhesion molecules of the L1 family sculpt functional cortical circuits during postnatal maturation in the mouse neocortex. Sema3 receptor complexes comprising L1-CAMs, Neuropilins (Npn1/2) and PlexinA subunits (PlexA1-4) associate in different combinations to drive elimination of distinct spine subpopulations in an activity dependent manner. Sema3F induces spine pruning through NrCAM, Npn2, and PlexA3 [Mohan et al., Cer.Cor. 29: 963, 2019], while Sema3B induces pruning through Close Homolog of L1 (CHL1), Npn2, and PlexA4 [Mohan et al., J. Neurosci. 39: 6233, 2019]. We focused on Sema3F signaling through NrCAM to elucidate signal transduction mechanisms of spine pruning. Structure-function studies revealed that NrCAM stabilizes binding between Npn2 and PlexA3 necessary for Sema3F receptor clustering and PlexA3 signaling in mouse cortical neurons in culture. Using immunofluorescence and confocal image analysis of individual spines, we identified a dual signaling pathway that drives Sema3F-induced pruning through Tiam1-Rac1-PAK1-3-LIMK1/2-Cofilin1 and RhoA-ROCK1/2-Myosin II. We suggest that the RhoA-ROCK1/2-Myosin II pathway generates contractile force that exerts tension on actin filaments assembled through the Rac1 pathway leading to F-actin catastrophe and spine collapse. Inhibition of spine collapse by latrunculin A and jasplakinolide supports a role for actin cytoskeletal remodeling in spine elimination. We have also used 2 mouse genetic models to demonstrate that L1, the family prototype, mediates spine pruning. Mouse mutants deleted for L1 or with a mutation in the binding motif for actin adaptor Ankyrin B display increased spine density and increased proportion of immature spines on apical dendrites in the prefrontal and primary visual cortex. Additionally, conditional knockout of Ankyrin B in cortical pyramidal neurons induced at early postnatal stages in a novel mouse mutant (Nex1-Cre-ERT2: Ank2 F/F: RCE) causes a phenotype of elevated spine density and immature spines, which persists in adulthood. Understanding the molecular events governing developmental spine pruning contributes to our understanding of how neuronal networks are sculpted and provides insight into how their dysregulation can lead to spine dysgenesis in diseases such as autism and schizophrenia.
Parallel Processing of Two Mechanosensory Modalities by a Single Neuron in *C. elegans*

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Neurons convert synaptic or sensory inputs into cellular outputs. It is not well understood how a single neuron senses, processes multiple stimuli, and generates distinct neuronal outcomes. Here, we describe the mechanism by which the *C. elegans* PVD neurons sense two mechanical stimuli: external touch and proprioceptive body movement. These two stimuli are detected by distinct mechanosensitive DEG/ENaC/ASIC channels, which trigger distinct cellular outputs linked to mechanonociception and proprioception. Mechanonociception depends on DEGT-1 and activates PVD’s downstream command interneurons through its axon, while proprioception depends on DEL-1, UNC-8, and MEC-10 to induce local dendritic Ca²⁺ increase and dendritic release of a neuropeptide NLP-12. NLP-12 directly modulates neuromuscular junction activity through the cholecystokinin receptor homolog on motor axons, setting muscle tone and movement vigor. Thus, the same neuron simultaneously uses both its axon and dendrites as output apparatus to drive distinct sensorimotor outcomes.

An autoinhibitory clamp of actin assembly promotes synaptic endocytosis

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Synaptic membrane-remodeling events such as endocytosis require force-generating actin assembly. The endocytic machinery that regulates these actin and membrane dynamics localizes at high concentrations in a micron-scale synaptic membrane domain called the ‘periactive zone’ (PAZ). Despite this high concentration of endocytic-actin regulators, we find that endocytic events occur only sparsely in space and time within the PAZ. To identify that mechanisms that constrain activation of this machinery in space and time, we combined *in vitro* analyses of membrane binding and actin assembly with *in vivo* analysis of actin dynamics and endocytosis at the *Drosophila* neuromuscular junction. We identify a mechanism whereby intramolecular autoinhibition clamps the PAZ machinery to limit actin assembly to discrete functional events: We found that collective interactions between the *Drosophila* PAZ proteins Nwk/FCHSD2, Dap160/Intersectin, and WASp relieve Nwk autoinhibition and promote collective recruitment to negatively charged membranes *in vitro*. By pyrene-labeled actin assembly assays, we find that these same collective protein and lipid interactions are essential for robust actin assembly. Together, this collective relief from autoinhibition constrains active actin assembly to the membrane surface. These same mechanisms appear to regulate endocytic actin dynamics at the synapse *in vivo*: We find by FRAP and SIM microscopy that the interaction between Nwk and Dap160 promotes membrane association and colocalization at the *Drosophila* neuromuscular junction synapse. Finally, by automated particle tracking to quantify synaptic actin dynamics *in vivo*, we discovered that Nwk-Dap160 interactions constrain spurious assembly of WASp-dependent actin structures, as loss of Nwk, Dap160, or the Nwk-interacting domain of Dap160 each cause a significant increase in the
frequency of brief (<30sec) actin patches. By measuring FM dye uptake and release, we find that these same manipulations also compromise synaptic endocytosis, suggesting that autoinhibition both clamps and primes the synaptic endocytic machinery, thereby constraining actin assembly to drive productive membrane remodeling in response to physiological cues.

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Shootin1a-mediated Actin-adhesion Coupling Generates Force To Trigger Structural Plasticity of Dendritic Spines

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Dendritic spines are tiny actin-enriched protrusions emanating from dendritic shafts that constitute the major compartments of excitatory post-synapses. They undergo activity-dependent structural plasticity, which is thought as a key mechanism to change the synaptic efficacy underlying learning and memory. The change in size and shape of dendritic spines requires actin dynamics. It has been shown that actin filament (F-actin) polymerizes at the tip of spines and undergoes retrograde movement. In addition, cell adhesion molecules and extracellular adhesive substrates are reported to be involved in the formation and plasticity of spines. However, how the mechanical force to induce spine enlargement is generated and molecular machinery that suffices for activity-dependent spine structural plasticity are unclear. Shootin1a is a brain-specific clutch molecule involved in the neuronal polarity formation and axon guidance. In this study, we found that shootin1a is localized at the tips of dendritic filopodia, stubby spines, thin spines and mushroom spines. To examine whether shootin1a is involved in dendritic spine formation, we performed shootin1 knock down analysis. Number of dendritic spines and filopodia were decreased by shootin1a-RNAi. Our in vivo data also revealed that hippocampal neurons in shootin1-KO mice brain exhibited a low number of dendritic spines and filopodia. Immunocytochemical analysis showed that shootin1a is co-localized with cortactin, F-actin, and L1-CAM in dendritic filopodia. Interestingly, N-cadherin was also found to be localized at the extrasynaptic sites of dendritic spines. In vitro binding analysis revealed that shootin1a interacts directly with N-cadherin. Single speckle molecule imaging showed that the velocity of F-actin retrograde flow in dendritic filopodia was increased when the shootin1a–L1-CAM or shootin1a–N-cadherin interaction was disrupted by shootin1a dominant negative mutants. Notably, the traction force generated by dendritic filopodia was also decreased by shootin1a dominant negative mutant. In addition, disruption actin-adhesion coupling by the dominant negative mutant inhibited the formation of dendritic spines and filopodia. These data indicate that shootin1a-mediated actin-adhesion coupling generates force for dendritic spines formation. Furthermore, induction of synaptic activation with glycine treatment (chemical LTP) enhanced shootin1a-mediated actin-adhesion coupling in spines. Finally, downregulation of shootin1a by RNAi inhibited the spine enlargement during glutamate uncaging stimulation in brain tissue. Taken together, these results suggest that shootin1a-mediated actin-adhesion coupling generates force to trigger structural plasticity of dendritic spines.
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Sucrose binge drinking predicts oxycodone conditioned place preference and modifies ProBDNF levels in brain reward areas

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Introduction: The effect of adolescent diet on predisposition to oxycodone abuse in adults remains unknown. Brain Derived Neurotrophic Factor (BDNF) regulates opioid induced-plasticity in the mature brain; thus, may be pivotal in linking food intake and oxycodone responses. **Aims:** 1) determine the effects of adolescent sucrose binge drinking (SBD) in oxycodone-induced conditioned place preference (CPP); 2) Assess SBD effects on levels of Pro-BDNF/Mature-BDNF in brain reward areas. **Methods:** Sucrose binge drinking model: from PND28-31 adolescent rats had limited (2 hr/day) access to sucrose (0.14M or 0.44M/day). On PND90 adult rats were tested for CPP after being randomly assigned to drug group (four daily 20-min pairings with oxycodone (3 mg/kg, s.c.) and four daily pairings with saline on alternate days, or to the control group (20-min pairings with saline each day). Following conditioning, rats received a 20-min CPP-test. After the preference test, brains were collected and analyzed for Pro and Mature BDNF levels. **Results:** oxycodone induced CPP [F (1, 21) = 55.02, p < .001], and CPP was significantly greater after SBD [F (1, 21) = 5.31, p < .05]. Oxycodone induced-CPP increased levels of ProBDNF in the prefrontal cortex [F (1, 21) = 14.46, p < .001] and Nucleus accumbens [F (1, 21) = 7.82, p < .01]. Does SBD alter the effect of oxy? Adolescent SBD enhanced oxycodone CPP and modified ProBDNF levels in brain reward areas in adults. Thus, adolescent diet may affect cellular mechanisms associated with reward and, thereby, alter responses to drugs of abuse in adults.

Tumor Invasion and Metastasis: Tumor Progression

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TMBIM6/BI-1 contributes to cancer progression through assembly with mTORC2 and AKT activation

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Transmembrane B cell lymphoma 2-associated X protein inhibitor motif-containing (TMBIM) 6, a Ca²⁺ channel-like protein, is highly upregulated in several cancer types. Here, we show that TMBIM6 is closely associated with survival in patients with cervical, breast, lung, and prostate cancer. TMBIM6 deletion or knockdown suppressed primary tumor growth. Further, mTORC2 activation was up-regulated by TMBIM6 and stimulated glycolysis, protein synthesis, and the expression of lipid synthesis genes and glycosylated proteins. Moreover, ER-leaky Ca²⁺ from TMBIM6, a unique characteristic, was shown to affect mTORC2 assembly and its association with ribosomes. In addition, we identified that BIA compound, a suggestive TMBIM6 antagonist, prevented TMBIM6 binding to mTORC2, decreased mTORC2 activity, and also regulated TMBIM6-leaky Ca²⁺, further suppressing tumor formation and progression in cancer xenograft models. This previously unknown signaling cascade in which mTORC2 activity is enhanced via the interaction with TMBIM6 provides effective therapeutic targets for various malignancies.
Mechanism and impact of Bone morphogenetic proteins (BMP)-mediated Sox2 downregulation in Ovarian Cancer

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BACKGROUND: Bone morphogenetic proteins (BMPs) are the largest subgroup of the Transforming Growth Factor-β (TGFβ) superfamily controlling diverse cellular responses and like TGFβ, exhibit dichotomous roles in cancer. Previously, we demonstrated that BMP9/GDF2 is significantly reduced in expression in ovarian cancer (OVCA) cells, methylated in patient tumors, and promoted anoikis resistance in both breast and OVCA cell lines. In an attempt to identify genes downstream of BMP9 that may provide anoikis resistance, transcriptomics was performed leading to the identification of Sox2, a developmental gene with prior established roles in OVCA, as being significantly downregulated in response to BMP9. The transcription factor Sox2 is a commonly amplified and overexpressed gene in OVCA. Sox2 is necessary for the maintenance of pluripotency and self-renewal with a reciprocal relationship between BMP's and Sox2 seen during various developmental processes. The studies presented here begin to elucidate the mechanisms of BMP-mediated Sox2 regulation in the context of ovarian cancer, which is the most lethal gynecologic malignancy in women due to peritoneal metastatic spread.

METHODS, RESULTS AND CONCLUSIONS: We validated our transcriptomics findings and examined the effect of the broader BMP family members on Sox2 expression at the protein and RNA levels in a spectrum of cancer cell lines. We find that BMP members including BMP2, BMP4, BMP9 but not BMP10 both in a time and dose dependent manner broadly suppress Sox2 expression. To test if Sox2 repression occurs via SMAD activation, we used inhibitors to the ALK receptors and find that SMAD1 activation is required for BMP-mediated Sox2 downregulation. This was validated via SMAD-sh and siRNA-based approaches. To test the involvement of the Sox2 promoter in its repression by BMP’s, we performed reporter luciferase assay using a 1kb Sox2 promoter. We also confirmed the role of the Sox2 promoter by expressing Sox2 from a heterologous CMV promoter, which failed to be repressed by BMP’s. Furthermore, we find that de novo protein and RNA synthesis is required for BMP-mediated Sox2 downregulation as determined using cycloheximide and ActinomycinD (protein and RNA synthesis inhibitors). To examine if DNA methylation and/or histone modifications are involved in BMP-mediated Sox2 repression, we used DNMT modifiers and HDAC inhibitors and find specifically that DNA methylation is required for BMP-mediated Sox2 repression. Using Chromatin Immunoprecipitation assay we provide evidence of SMAD1 binding to Sox2 promoter in response to BMP. The role of BMP-mediated Sox2 repression on OVCA cell survival, metastasis, and anoikis and the specific repression mechanism involved will also be presented.

Nuclear deformation causes DNA damage by increasing replication stress

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Cancer metastasis, i.e., the spreading of tumor cells from the primary tumor to distant organs, is responsible for the vast majority of cancer deaths. In the process, cancer cells migrate through narrow
interstitial spaces substantially smaller in cross-section than the cell. During such confined migration, cancer cells experience extensive nuclear deformation, nuclear envelope rupture, and DNA damage. The molecular mechanisms responsible for the confined migration-induced DNA damage remain incompletely understood. While in some cell lines, DNA damage is closely associated with nuclear envelope rupture, we show that in others, mechanical deformation of the nucleus is sufficient to cause DNA damage, even in the absence of nuclear envelope rupture. This deformation-induced DNA damage, unlike nuclear envelope rupture-induced DNA damage, occurs primarily in S/G2 phase of the cell cycle and is associated with stalled replication forks. Nuclear deformation, resulting from either confined migration or external cell compression, increases replication fork stalling and replication stress, providing a molecular mechanism for the deformation-induced DNA damage. Thus, we have uncovered a new mechanism for mechanically induced DNA damage, linking mechanical deformation of the nucleus to DNA replication stress. This mechanically induced DNA damage could not only increase genomic instability in metastasizing cancer cells, but could also cause DNA damage in non-migrating cells and tissues that experience mechanical compression during development, thereby contributing to tumorigenesis and DNA damage response activation.

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**Enrichment of the mevalonate pathway in cooperatively invading cells underlies phenotypic heterogeneity in breast cancer invasion**

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Intratumor heterogeneity poses a significant hurdle for cancer treatment, yet is under-characterized in the context of metastasis. Cancer cells from human solid tumors can invade through two predominant modes: collective invasion, whereby cancer cells invade in multi-cellular packs or streams marked by intact cell-cell junctions; and single-cell invasion, whereby cells invade independently without intercellular adhesion. Both collective and single-cell invasion co-occur within the same tumor microenvironment, suggesting that an intrinsic program exists to oversee this phenotypic heterogeneity. To delineate this program, we used a novel published technique developed by the lab (SaGA) to isolate pure subpopulations of 4T1 cells that collectively invade (cooperators) or single cells that invade alone (singles). 3-D spheroids of SaGA-purified cooperators and singles embedded in collagen exhibit almost exclusively collective and single-cell invasion, respectively, and these invasive phenotypes are retained over multiple passages. Gene set enrichment analysis of bulk RNAseq data obtained from RNA isolates of cooperators and singles highlighted the enrichment of the cholesterol biosynthesis pathway in cooperators. RNA transcripts of all but one enzyme in the cholesterol biosynthesis pathway were significantly over-expressed in cooperators when compared to singles. However, cooperators and singles showed no significant differences in free cholesterol levels, suggesting an alternative output of the cholesterol biosynthesis pathway that differentiates collective versus single-cell invasion. Notably, farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) are intermediates of the cholesterol biosynthesis pathway that are substrates required for prenylating key proteins that drive invasion and metastasis, including Ras and Rho kinase. Indeed, RNA transcripts of key prenylation enzymes FNTA, RABGGTA and RABGGTB were significantly over-expressed in cooperators when compared to singles. Together, our data suggest that differential prenylation patterns may be crucial in driving invasive phenotypic heterogeneity in metastatic cancer cells.
Characterization of equol production in a cohort of Puerto Rican women

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The US Hispanic female population has one of the highest breast cancer (BC) incidence and mortality rates, while BC is the leading cause of cancer death in Puerto Rican women. Nutrition is a cancer contributing factor, and our previous studies indicate that consuming combined soy isoflavones (genistein, daidzein, and glycitein) promotes cell proliferation and tumor metastasis through increased protein synthesis. We further demonstrated that the daidzein derived secondary metabolite, equol, is one of the main contributors for BC progression. Equol is produced in 30-50% of the US population that exhibit specific intestinal equol producing bacteria, which correlates with bean and soy consumption. Equol biosynthesis involves chemical modifications that may produce additional unidentified metabolites. Thus, we hypothesize that Puerto Rican women are efficient equol producers due to high bean consumption. To address our hypothesis, we conducted a cross-sectional characterization of equol production in a clinically based sample of healthy 25-50 year old Puerto Rican women (n=80). Urine samples were collected and evaluated for presence of soy isoflavones and metabolites to determine the ratio of equol producers to non-producers. Urine samples were also analyzed for new metabolites that could be potentially involved in equol biosynthesis. A gas chromatography/mass spectrometry (GC/MS) analysis was employed for urine metabolite and identification. GC/MS data analysis revealed seven urine metabolites including O-demethylangolensin (ODMA), Daidzein (Da), genistein (Ge), dehydrodaidzein (DHD), enterodiol (End), enterolactone (EnL), and equol. Our results indicate that Da and Ge are present in 95% and 91% of our cohort, respectively, while 25% are equol producers. Surprisingly, equol only correlated to ODMA (Da metabolite), End (lignin), and Ge (a soy isoflavone) (P<0.0001). These findings indicate that equol biosynthesis in Puerto Rican women may be related to other isoflavones aside from Da. Next, we will characterize bacteria that are responsible for equol production in this cohort of healthy Puerto Rican women in order to set the path for future studies to characterize and correlate equol production and metastasis in BC patients. The project was sponsored by NIH/NIGMS (SC3GM111171, MMM), NIH/NIMHD (8G12MD007583, UCC), NIH/NIMHD (8U54MD007587, UPR) grants.

Overexpression Of Nuclear Envelope Proteins In Metastatic Melanoma Promote Loss Of Nuclear Envelope Integrity During Confinement

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Metastatic melanoma is an aggressive disease, characterized by its high level of mutational burden. During metastasis, cells must migrate through tissue regions of high confinement, resulting in nuclear
deformation and loss of nuclear envelope (NE) integrity, generating double strand breaks in the DNA, improper repair of which is believed to result in heritable genomic aberrations. We hypothesize that during metastatic progression, the expression levels of genes encoding NE proteins are altered, promoting nuclear deformability and envelope fragility, thus driving genomic plasticity. To determine if metastatic melanoma cells are inherently more prone to rupture under confinement than their benign counterpart melanocytes, we utilized a PDMS device to transiently confine the cells and found under 3 µm confinement 35.4% of melanoma cell line 1205Lu exhibit DNA exposure to the cytosol, compared to only 10.1% of melanocytes. Utilizing atomic force microscopy (AFM) to measure the force required to rupture the nuclear membrane, we found 1205Lu cells ruptured easily around 150 nN while melanocytes were resistant to NE rupture up to 450 nN. Similar trends in nuclear rupture were also observed in cells migrating within tumor spheroids embedded in collagen to model more physiological 3d confinement. To determine the changes in NE gene expression during disease progression, we compared RNA-seq transcriptomic data sets from patient tumor samples of metastatic melanoma and benign nevi, metastatic melanoma cell lines, and primary human melanocytes, focusing on a subset of NE proteins transcriptionally upregulated in metastatic disease. Performing a targeted siRNA-based screen to assay for nuclear fragility, we found that reduction of Lamin B Receptor (LBR) dramatically reduced DNA exposure to the cytosol in 1205Lu cells. Furthermore, ectopic overexpression of LBR was sufficient to increase nuclear envelope fragility and DNA exposure to the cytosol in melanocytes. To determine if this increase in nuclear fragility was due to alterations in nuclear mechanics induced by LBR level, we utilized AFM to analyze nuclear stiffness, finding that overexpression of LBR was sufficient to reduce nuclear stiffness in melanocytes. These results show that upregulation of LBR, as seen in metastatic melanoma, promotes confinement-induced NE rupture, and plays a role in reducing the intrinsic stiffness of the nucleus, potentially promoting genetic heterogeneity in metastatic melanoma during migration in confined microenvironments. We are currently determining the molecular mechanism by which LBR promotes nuclear fragility.

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Towards Development of Biophysical Markers for Predicting Ovarian Cancer Progression

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Ovarian cancer presents the highest mortality rate of any gynecological cancer with ~54% of patients succumbing to the initial or recurrent diagnosis. Unfortunately, current diagnostic tools are either unable to detect the cancer before it has invaded the fibrous sub-mesothelium or need invasive techniques. Thus, there is a critical need for the development of alternative diagnostic tools that can significantly improve detection and increase the patients’ survival. Here, we identify a series of biophysical metrics as high throughput, rapid, and low-cost complementary diagnostic option to evaluate the metastatic potential in ovarian cancer cells at single-cell resolution on a timescale of a few hours. Specifically, we investigated if ovarian cancer progression in a mouse ovarian surface epithelial (MOSE) model can be correlated to cell shape, protrusive and migration dynamics, and contractility. To quantify these biophysical metrics in a precise and repeatable manner, we fabricated controlled fibrous architectures mimicking the fibrous sub-mesothelium using the non-electrospinning Spinneret-based Tunable Engineered Parameters (STEP) method: crosshatch networks of mismatch diameters for studying protrusion dynamics, aligned and crosshatch networks of same diameter but varying inter-fiber
spacing for studying cell migration, and finally, aligned nanonets for measuring cell forces. We found that both the protrusion length and \textit{coiling} (wrapping around the fiber axis) dynamics at the tip of the protrusion showed a positive correlation with disease progression in the MOSE model while migration dynamics showed a substrate dependent correlation with disease progression. Interestingly, the contractility followed a biphasic relationship with disease progression with the benign phenotype exhibiting the largest forces. This is primarily due to the sequential disorganization of the filamentous actin network with disease progression across the MOSE model. In summary, we have identified protrusive and \textit{coiling} dynamics as reliable biophysical metrics which can be used in conjunction with migration and force read-outs to potentially pinpoint the disease stage in a matter of hours and thus provide a complementary tool to current standard of care ovarian cancer diagnostics.

**Ubiquitin and Proteasome Function**

**P318**

\textbf{A selective transmembrane recognition mechanism by a membrane-anchored ubiquitin ligase complex}

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While it is well-known that E3 ubiquitin ligases can selectively ubiquitinatemembrane proteins in response to specific environmental cues, the underlying mechanisms for the selectivity are poorly understood. In particular, the role of transmembrane regions, if any, in target recognition remains an open question. Here, we describe how Ssh4, a yeast E3 ligase adaptor, recognizes the PQ-loop lysine transporter Ypq1 only after lysine starvation. We show the binding site is formed between two transmembrane helices of Ypq1 (TM5 and TM7) and the single transmembrane helix of Ssh4. This interaction is regulated by the conserved PQ motif. Strikingly, recent structural studies of the PQ-loop family have suggested that TM5 and TM7 undergo major conformational changes during substrate transport, implying that transport-associated conformational changes may determine the selectivity. These findings thus provide critical information concerning the regulatory mechanism through which transmembrane domains can be specifically recognized in response to changing environmental conditions.

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\textbf{Site-specific O-GlcNAc Modification is a Regulatory Bridge between Kelch-like Proteins and Intermediate Filaments}

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The Kelch-like (KLHL) protein family, a conserved group of 42 human members, binds to E3 ubiquitin ligase complexes to target protein substrates to the ubiquitin-proteasome system. Mutations in KLHL genes compromise proper protein substrate turnover, resulting in pathological protein aggregations in various human diseases. However, the regulation of KLHL function remains understudied. Post-translational modifications influence protein functions, yet how they govern KLHL proteins is not fully
explored. We have addressed this knowledge gap by studying the modification of KLHL proteins by O-linked-β-N-acetylglucosamine (O-GlcNAc). Governed by O-GlcNAc transferase (OGT) and O-GlcNACcase, this form of intracellular glycosylation decorates serine (S) or threonine residues of OGT substrates in response to cellular stimuli. Our previous work characterized the roles of two O-GlcNAcylation sites on gigaxonin, a KLHL protein, in facilitating the degradation of intermediate filament (IF) proteins, vimentin and neurofilament light (NF-L). Loss-of-function gigaxonin mutations cause IF accumulation in neurons, leading to axonal swelling and degeneration in the disease giant axonal neuropathy. Notably, both vimentin and NF-L are modified by O-GlcNAc themselves. We discovered vimentin O-GlcNAcylation on S49 to be essential for numerous functions: its homotypic association, normal IF morphology and cell migration. Interestingly, compared to wild type vimentin, the O-GlcNAcylation-deficient S49A mutant is a poorer substrate for gigaxonin-mediated degradation. This finding implies a potential regulatory bridge between KLHL and IF proteins by O-GlcNAc signaling. In addition, our preliminary results suggest that OGT overexpression may redistribute NF-L into lower-order assembly states and enhance its O-GlcNAcylation significantly, suggesting a control of equilibrium among NF-L assembly states by O-GlcNAc. Taken together, our current research proposes O-GlcNAcylation as a key regulator for IF assembly, degradation and functions. Ongoing work will identify functionally important O-GlcNAcylation residues of human NF-L in homeostasis and disease models. Our long-term goal is to define the role of site-specific O-GlcNAcylation as a functional link between KLHL and IF proteins in health and pathology.

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Regulation of Translocon Quality Control by a Heterodimeric Transcription Factor Complex


Proteins contribute to every cellular function. When proteins behave aberrantly, they are often degraded by cellular quality control mechanisms to prevent organismal harm. Proteins may behave aberrantly by persistently engaging (i.e. clogging) the translocon, a channel that allows proteins to move across the endoplasmic reticulum membrane. One protein known to clog translocons in humans is apolipoprotein B, a component of low-density lipoproteins. In humans and yeast, homologs of the Hrd1 ubiquitin ligase polyubiquitylate translocon-clogging proteins, tagging them for proteasomal degradation. Ubiquitin ligases rarely work alone; many function with protein co-factors. Further, yeast lacking Hrd1 exhibit residual degradation of model translocon-clogging proteins, suggesting alternative degradation pathways. We performed a yeast genome-wide screen to identify genes that may contribute to degradation of translocon-clogging proteins. We identified 150 genes in the screen. Small-scale reporter assays were performed, confirming potential roles for 42 genes. The confirmed genes were biochemically validated by cycloheximide chase to directly assess roles in protein degradation. Deletion of genes encoding both components of a heterodimeric transcription factor complex stabilized a model translocon-clogging protein. Whether these transcription factors specifically regulate translocon quality control or protein quality control broadly, and the relevant transcriptional targets of these transcription factors, are the subjects of ongoing investigation. Because translocation and translocon quality control are conserved in yeast and humans, these transcription factors represent potential therapeutic targets for patients with elevated levels of cholesterol.
Pa28γ Plays a role in regulating apoptosis following genotoxic stress  
E. Coffman, E. Aller, A. Grouls, A. Gunter, A. Sliz, N. Iqbal, R. Jamshidi, L. Barton; Austin College, Sherman, TX.

Proteasomes are multi-catalytic proteases that regulate various processes in the cell including mitosis and apoptosis. Proteasome activators, such as PA28γ, have been found to be highly expressed in cancerous cells with apoptotic resistance. Murine Embryonic Fibroblasts (MEF’s) lacking PA28γ are more susceptible to spontaneous apoptosis, suggesting PA28γ plays a role in programmed cell death. To determine how PA28γ affects the caspase-9 mediated apoptotic signaling pathway, MEF’s deficient in PA28γ were induced to enter apoptosis by various cytotoxic stimuli. PA28γ did not demonstrate a consistent pro- or anti-apoptotic role, with PA28γ sensitizing the MEFs to some stimuli and conferring resistance to others. These variable roles for PA28γ indicate that it plays a role upstream of the apoptosis pathway’s commitment step. Notably, out of the tested stimuli, PA28γ only seemed to regulate apoptosis in those treatments which are known to induce DNA damage, suggesting that PA28γ may induce or inhibit apoptosis primarily via management and repair of DNA damage. Furthermore, treatment with a proteasome inhibitor resulted in a decrease of apoptosis and the elimination of differences in the amount of apoptosis between PA28γ +/- and PA28γ -/- cells. These data suggest that PA28γ regulates apoptosis in a proteasome-dependent mechanism. These findings suggest that inhibiting PA28γ may be a promising target to enhance the effects of some chemotherapies, but this benefit will be dependent on both the mechanism of action and cellular characteristics.

Emerging role of kelch-like proteins as regulators of the UPR signaling  
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The Kelch-like gene family encodes a large group of proteins characterized by the presence of a BTB domain, a BACK domain and a Kelch repeat domain. The BTB domain is used to bind cullin3 (CUL3) RING ubiquitin ligase and form an active ubiquitin ligase complex, while the Kelch repeat domain is responsible for the interaction and transfer of ubiquitin to target substrates. Through their substrate-binding activity, Kelch-like proteins participate in a variety of biological processes including cytoskeleton organization, cell morphology and extracellular communication. Here we report a new biological role of Kelch-like proteins as new regulators of the unfolded protein response (UPR). The UPR signaling pathway is activated upon endoplasmic reticulum (ER) stress and it is crucial for reestablishing protein homeostasis. The UPR is transduced via three sensors: pancreatic ER eukaryotic translation initiation factor (eIF)-2 α kinase (PERK), inositol-requiring enzyme 1 (IRE-1), and activating transcription factor 6 (ATF6). A knockdown or overexpression of the Kelch-like proteins KLHL12 and KLHL41 triggers a dysregulation of the UPR in different cells lines. Additionally, KLHL40 knockdown resulted in a dysregulation of the PERK branch in C2C12 cells, corroborating the importance of Kelch-like proteins in the regulation of the UPR. KLHL41 and KLHL40 mutations cause Nemaline Myopathy (NM), a congenital disorder that typically shows weakness and growth defects primarily in the skeletal muscle. A prevailing model suggests that KLHL40 and KLHL41 regulate the stability of the sarcomeric thin filaments. As our preliminary data point to a new role for KLHL40 and KLHL41 in the UPR and the UPR has been implicated...
in muscle growth, we are proposing that UPR dysregulation imposed by defects in KLHL40 or KLHL41 is responsible for muscle growth defects in NM. We generated \textit{klhl41} mutants in zebrafish. The \textit{klhl41} mutants showed an extensive disarray of myofibers, impaired swimming, pericardial edema and premature death. Our experiments are now focused on understanding the mechanism by which those Kelch-like proteins affect the UPR and its implication on muscle dysfunction during NM pathogenesis.

**Monday, December 14, 2020, 1:00 pm**

**Actin Cytoskeleton in Organelle Morphology and Dynamics**

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**The Birth and Death of a Microvillus**

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Actin-based protrusions are one of the defining features of the eukaryotic cell surface, conferring cells with the ability to move and interact with the local environment. Central to the specialization of animal cells is the emergence of microvilli, parallel actin-based protrusions that line the apical surface of epithelial cells. Microvilli in transporting epithelia consist of a core bundle of 20-30 actin filaments wrapped in membrane, with membrane-cytoskeleton linkers stabilizing this basic morphology. Transmission electron microscopy studies showed that filaments in core bundles are oriented with their barbed ends towards the distal tips. These micrographs also revealed an electron dense plaque at the distal tips, presumed to contain barbed-end targeting proteins that control the growth of microvilli actin filaments. Although recent studies have begun to uncover factors that control microvillus organization and morphology, the molecular events that underlie the growth of microvilli \textit{de novo} are poorly understood, and the growth of individual microvilli has not been captured via live cell imaging. Using spinning disc confocal microscopy and the kidney proximal tubule LLC-PK1-CL4 (CL4) cell line as a model system, we sought characterize individual microvillus growth events. We found that the apical surface of early differentiated CL4 cells undergoes dramatic remodeling, with microvilli both growing and collapsing. Microvillus growth and collapse are typified by a series of molecular events, with distal tip targeting proteins EPS8 and IRTKS localized to sites of microvilli growth before the F-actin bundle marker Espin, while the localization of the membrane-cytoskeleton linker Ezrin coincides with actin bundle formation. Conversely, collapse of microvilli is characterized by loss of EPS8 and IRTKS from the distal tips, which is preceded by co-incident loss of Ezrin and plasma membrane protrusion. Using these quantitative time-lapse datasets, we present a framework whereby distal tip targeting proteins serve as early signals for sites of microvilli growth, followed by recruitment of actin bundling proteins, core bundle formation and coincident plasma membrane wrapping with the help of membrane-cytoskeleton linkers. These studies offer the first temporally resolved microvilli growth mechanism and highlight key molecules and activities that function in this capacity. These findings also hold implications for understanding the growth of other classes of surface protrusions, including filopodia and stereocilia.
Acute actin assembly on depolarized mitochondria mediated by Arp2/3 complex and WAVE inhibits Parkin-mediated mitophagy

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Mitochondrial depolarization, induces a number of cellular responses including actin polymerization and mitophagy through the PINK1/Parkin pathway. For actin, two distinct bursts of polymerization occur after depolarization (Kruppa et al (2018) Dev. Cell 29398621), which we define as: 1) ADIA (acute depolarization-induced actin), which initiates within 2 min and disappears by 10 min; and 2) PDIA (prolonged depolarization-induced actin), which occurs after 1 hr of depolarization. Here, we focus ADIA (acute depolarization-induced actin). While ADIA requires Arp2/3 complex (Fung et al (2019) J. Cell Sci. 31413070), other actin binding proteins involved in ADIA are unknown. Furthermore, the consequences of ADIA are unclear. We show that Arp2/3 complex is activated by the nucleation promoting factor (NPF) WAVE during ADIA, and that other NPFs (WASH, WHAMM, JMY, N-WASP and cortactin) are not involved. In this manner, ADIA is distinct from PDIA, which requires the NPF N-WASP. We also find a formin requirement for ADIA, specifically for the FMNL family, with the three FMNLs being redundant for this purpose. Our model is that FMNL formins produce the mother filaments for Arp2/3 complex-induced branched actin assembly during ADIA. The purpose of ADIA is unknown. Mitochondrial depolarization is known to cause Parkin recruitment, with subsequent Parkin-mediated ubiquitination resulting in mitophagy. We find that inhibition of ADIA accelerates Parkin recruitment to depolarized mitochondria. In control U2OS cells, Parkin recruitment starts at 45.4 min ± 10.7 min (mean ± s.d.), while CK666 treatment (inhibiting Arp2/3 complex), FMNL suppression or WAVE complex suppression accelerates Parkin recruitment (29.4 min ± 9.0 min, 32.6 min ± 8.7 min and 33.4 ± 10.3 min respectively). These results suggest a model whereby ADIA transiently delays mitophagy, allowing time for mitochondrial recovery before they are targeted for PINK1/Parkin mitophagy. Overall, the two successive rounds of depolarization-induced actin polymerization have opposite effects, with ADIA delaying mitophagy and PDIA enhancing mitophagy. Our results point to an intricate relationship between actin polymerization and mitochondrial function, with the function of actin depending on the time it is polymerized.

Non-muscle myosin II and fascin organize the INF2-mediated cytoplasmic actin network, with downstream effects on mitochondria

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Non-muscle myosin II (NMII) is a hexameric actin-binding protein, that can self-assemble into bipolar filaments, which in conjunction with F-actin exert cellular contractile forces. There are three NMII proteins in mammals, with NMIIA (Myh9 gene) being important for leading edge dynamics, adhesion formation and cellular contractility, NMIB (Myh10) being important for maintaining focal adhesions and
cellular front-rear polarity, and NMIIC (Myh14) being less well characterized. Here, we investigate the role for NMII members in regulating mitochondrial dynamics. We initially found that treatment with several myosin II inhibitors caused extensive mitochondrial elongation in multiple cell lines. Thus, we sought to address the mechanism by which NMII affects mitochondria morphology and function. The three NMIIIs have unique kinetic properties and both specific and redundant cellular functions. In order to dissect their individual roles at mitochondria, we generated individual NMIIA, -IIB and -IIC CRISPR KOs in U2OS cells. Loss of any single NMII caused mitochondria to become elongated and hyperconnected, suggesting either an inhibition of mitochondrial division or a stimulation of mitochondrial fusion. We have previously shown that the ER-bound formin INF2 polymerizes a transient actin meshwork ('actin burst') facilitating ER-calcium transfer to mitochondria and subsequent division. Both the actin burst and mitochondrial calcium uptake was significantly hampered in all NMII KOs, suggesting that NMII members are involved in organizing and/or contracting these actin filaments to promote ER-mitochondria contacts. Interestingly, NMIIC KO had the strongest effect on mitochondrial calcium influx and the actin burst, although being the least expressed isoform in U2OS cells. We also show for the first time that NMIIIs transiently colocalize with the INF2-polymerized actin meshwork. Another question was whether additional proteins were required, along with INF2 and NMII, to organize the actin burst filaments into a network. One prime candidate was α-actinin4, since both INF2 and α-actinin4 mutations link with the kidney disease focal segmental glomerulosclerosis. Surprisingly, knockdown of α-actinin1 or α-actinin-4 had no effect on the actin burst. However, knockdown of the actin bundling protein fascin dramatically decreased actin burst density and altered the appearance of the filamentous network. We propose a mechanism in which INF2 polymerizes F-actin, that is crosslinked and stabilized by fascin and organized/contracted by NMII. Taken together, these actin-binding proteins work in concert to facilitate ER-mitochondrial interactions and efficient ER-to-mitochondrial calcium transfer, leading to mitochondrial constriction and division.

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A cryptic sequence targets the adhesion complex scaffold ANKS4B to apical microvilli to promote enterocyte brush border assembly

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Nutrient-transporting enterocytes interact with their luminal environment using a densely-packed collection of apical microvilli known as the brush border. Assembly of the brush border is controlled by the intermicrovillar adhesion complex (IMAC), a protocadherin-based complex found at the tips of brush border microvilli that mediates adhesion between neighboring protrusions. ANKS4B is known to be an essential scaffold within the IMAC, though its functional properties have not been thoroughly characterized. We report here that ANKS4B is directed to the brush border using a non-canonical apical targeting sequence that maps to a previously unannotated region of the scaffold. When expressed on its own, this sequence targeted to microvilli in the absence of any direct interaction with the other IMAC components. Sequence analysis revealed a coiled-coil motif and a putative membrane-binding basic-hydrophobic repeat sequence within this targeting region, both of which were required for the scaffold to target and mediate brush border assembly. Size exclusion chromatography of the isolated targeting sequence coupled with in vitro brush border binding assays suggests that it functions as an oligomer. We further show that the corresponding sequence found in the closest homolog of ANKS4B, the scaffold
USH1G that operates in sensory epithelia as part of the Usher complex, lacks the inherent ability to target to microvilli. This study further defines the underlying mechanism of how ANKS4B targets to the apical domain of enterocytes to drive brush border assembly, and identifies a point of functional divergence between the ankyrin repeat-based scaffolds found in the IMAC and Usher complex.

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**Actin polymerization around depolarized mitochondria disrupts ER-mitochondrial contact, delaying Parkin accumulation and mitophagy**

R. Chakrabarti, T. Fung, H. N. Higgs; Geisel School of Medicine at Dartmouth, Hanover, NH.

Actin polymerization around depolarized mitochondria disrupts ER-mitochondrial contact, delaying Parkin accumulation and mitophagy

Rajarshi Chakrabarti, Tak Shun Fung & Henry H. Higgs

A growing number of studies suggests that actin polymerization participates in mitochondrial communication and dynamics in mammalian cells, with multiple distinct modes of actin/mitochondria interaction. In one mode (Fung et al (2019) J. Cell Sci.), mitochondrial depolarization causes rapid accumulation of an extensive ‘shell’ of actin filaments around depolarized mitochondria, dependent on Arp2/3 complex. We call this ADIA (Acute Depolymerization-Induced Actin). Here, we show that ADIA requires WAVE complex for Arp2/3 activation, as well as contributions from FMNL family of formins. ADIA is temporally distinct from a second round of actin polymerization that occurs over 1 hr after depolarization (Kruppa et al (2018) Dev. Cell.), which we call PDIA (Prolonged Depolarization-Induced Actin). We show that ADIA does not require myosin 6, further differentiating it from PDIA. Mitochondrial depolarization results in several other events, including Parkin accumulation. ADIA precedes Parkin accumulation, with peak actin polymerization at 2 min whereas Parkin accumulation starts at 45 ± 10 min after depolarization. Surprisingly, inhibition of ADIA by either inhibition of Arp2/3 complex or FMNL knock-down accelerates Parkin accumulation to 29 ± 9 min and 32 ± 8 min, suggesting that ADIA might inhibit Parkin recruitment to mitochondria. Our initial hypothesis was that the actin shell might serve as a barrier to Parkin access. However, experiments using rapamycin-inducible recruitment of a fluorescent protein to the mitochondrial outer membrane did not support this model. Interestingly, we noticed that ADIA promotes detethering of ER from mitochondria upon mitochondrial depolarization, an effect not observed for interactions between mitochondria and lysosomes. To test the effect of ER-mitochondrial tethering on Parkin recruitment, we artificially increased tethering by expression of VAP-B. Increased ER-mitochondrial tethering accelerates Parkin accumulation on depolarized mitochondria to 24 ± 8 min, phenocopying the effect of Arp2/3 and FMNL inhibition. We propose that ADIA acts as a transient protective mechanism, allowing mitochondria a chance to recover polarity before mitophagic pathways are engaged. On a larger scale, our data show that the purpose of actin polymerization around depolarized mitochondria changes over time, with ADIA delaying mitophagy and PDIA facilitating mitophagy.
Actin and INF2 at the intersection of organelle morphology and motility
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The actin cytoskeleton plays important roles in a variety of cellular functions, including the regulation of organelle dynamics. In particular, the role of actin in the regulation of mitochondrial fission has been previously explored. Current evidence supports the model that actin polymerizes at ER-mitochondria contact sites, promoting mitochondrial constriction and allowing recruitment of key players in mitochondrial fission such as DRP1. Using probes we designed to specifically label mitochondria- or ER-associated actin, we were able to visualize accumulation of actin at mitochondrial fission sites, in agreement with already existing evidence. In addition, we made the novel observation that ER-associated actin also consistently accumulates at a wide range of other organelle fission sites including endosomes, lysosomes, peroxisomes, and the Golgi, suggesting a broad mechanism of organelle regulation by actin polymerization at ER-organelle contact sites. We further pursued INF2 as a potential regulator of this process. INF2 is a formin protein which promotes actin assembly. The ER-localized isoform of INF2 has been shown to regulate mitochondrial morphology and motility through its actin polymerization activity. We observed that INF2 deletion causes dramatic enlargement and tubulation of endosomes as well as lysosomal enlargement, suggesting that INF2 also plays a role in promoting endolysosomal fission, similar to its role in mitochondrial fission. Additionally, expression of a dominant active mutant of INF2 drastically reduces endosomal and lysosomal movement. This demonstrates that INF2 modulates not only organelle morphology but also organelle movement and this regulation is not limited to mitochondria.

Actin Dynamics during Development

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Regulation of Epithelial Morphogenesis by Actin Disassembly
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Epithelial morphogenesis is an essential process during the development. These processes require cells to intercept environmental cues resulting in actin nucleation, orchestration of contractile actomyosin filaments and communication via the cell-cell junctions. This is enabled by the existence of distinct filamentous actin networks, composed of contractile actomyosin fibers and protrusive networks, whose turnover is spatially regulated. How the differential dynamic properties of actin networks in epithelial cells are achieved, and how different actin-binding proteins are coordinated to promote actin disassembly remains to be elucidated. While cofilin is considered as a main actin severing and depolymerizing factor, also other actin-binding proteins have been implicated to assist cofilin in mediating actin disassembly in vitro; such as CAP (Cyclase-associated protein) and AIP1 (Actin-interacting protein 1). However, it is not fully understood how they orchestrate F-actin disassembly with cofilin and each other in vivo. Therefore, our objective is to understand the molecular mechanisms and spatiotemporal regulation of actin disassembly, and its specific cellular roles in cell migration, adhesion and epithelial integrity during development. We use Drosophila genetics, state-of-the art imaging and
image-analysis techniques to determine the nature of actin filament networks directed for disassembly, and the consequences of disrupted actin disassembly for cellular and cytoskeletal architecture and adhesion. Our results show that in follicular epithelium of Drosophila melanogaster both CAP loss-of-function and silencing of Aip1 results in increased actin accumulation in distinct cellular locations, thus suggesting they are both required for efficient actin turnover with specific functions.

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**Lipid droplets regulate actin dynamics via prostaglandin signaling during Drosophila oogenesis**

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Oocytes of many animal species have large amounts of lipid droplets which serve as an energy source for the developing embryo. In Drosophila, loss of the triglyceride synthesis enzyme DGAT1 causes sterility, and developing follicles display severe defects in actin remodeling and eventually die. We find that loss of three different lipid droplet-associated proteins (the triglyceride lipase ATGL, the anchoring protein Jabba, and the Perilipin PLIN2) supports oogenesis but results in an unusual actin phenotype that includes faulty actin bundle assembly, disrupted cortical actin, and a failure of contraction. These results suggest that lipid droplets serve an important regulatory function during oogenesis. This actin phenotype closely resembles that seen with loss of the cyclooxgenase-like enzyme Pxt, responsible for the production of lipid signals termed prostaglandins. Using dominant genetic interaction studies, we find that PLIN2 regulates actin remodeling independent of Pxt, whereas Jabba and Pxt, and ATGL and Pxt function in two distinct pathways to regulate actin. In mammalian cells, ATGL-mediated triglyceride breakdown can release arachidonic acid which subsequently serves as substrate for prostaglandin synthesis. We therefore hypothesize that in the ovary ATGL functions similarly upstream of Pxt. Preliminary lipidomics results indeed indicate the presence of arachidonic acid-containing triglycerides in ovaries. We are currently testing whether exogenous prostaglandin can restore actin remodeling and in vitro follicle development in atgl mutant follicles. However, unlike in mammalian cells where lipid droplets can serve as a platform for prostaglandin synthesis, Pxt does not reside on lipid droplets, but localizes to the Golgi and the endoplasmic reticulum (ER), suggesting that arachidonic acid released from the lipid droplets by ATGL is shuttled to the Golgi and ER for prostaglandin production. These studies are among the first connecting lipid droplets to actin cytoskeletal regulation and suggest that one of the molecular mechanisms for this connection involves modulation of prostaglandin signaling. As lipid droplets, prostaglandins, and actin all have clinically relevant roles in many diseases, including cancer, these studies may help identify new therapeutic targets.

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**Investigating mechanisms regulating actin organization in the early Drosophila embryo**

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During development, actin networks undergo many dynamic rearrangements that promote morphogenesis in the developing embryo. Therefore, actin network dynamics must be tightly regulated in space and time. Though we know the actin cytoskeleton is necessary for maintaining shape and integrity, it is not fully understood how actin networks are assembled and organized during...
morphogenetic events. Through a directed genetic screen of factors necessary for *Drosophila* gastrulation, we identified a synaptotagmin-like protein called Bitesize (Btsz) as a potential regulator of the actin network. Btsz-depletion in early *Drosophila* embryos resembled the effects of disrupting actin dynamics, destabilizing intercellular attachments and actin network integrity during this stage. This observation is in line with previous work that suggests Btsz functions in adherens junction stabilization. In addition, our preliminary data have identified a novel phenotype before gastrulation in embryos maternally depleted of Btsz by RNAi. This function occurs during syncytial blastoderm development, which takes place before adherens junctions have been established. During this stage of development, nuclei divide synchronously and ingestions composed of plasma membrane and actin, called metaphase furrows, form compartments to prevent mitotic spindle collision. Actin caps are F-actin (filamentous actin)-containing structures above syncytial nuclei, and expand and collide to help form these metaphase furrows. In Btsz-depleted embryos, actin cap growth is affected and metaphase furrows are shorter than those in wild type embryos. Cell division defects often occur as a result of shorter metaphase furrows and indeed, spindles collide in the absence of Btsz. Taken together, the data suggest that Btsz regulates actin in a more direct manner than through adherens junction stabilization. To determine how Btsz regulates actin, we biochemically tested Btsz activity. We found that the C-terminal half of Btsz binds F-actin with μM affinity. Moreover, we found that Btsz accelerates formin-dependent F-actin assembly *in vitro*. We are currently investigating the mechanism through which Btsz may regulate actin networks and formin activity *in vivo*.

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Neurite morphogenesis requires spatial coordination of actin protrusion and contractility by septin7

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Neuronal axons and dendrites develop from membrane protrusions of the neuronal cell body. Formation of neurites, the early precursors of axons and dendrites, involves the establishment of actin-rich filopodia from the circumferential lamellae of the cell body, and quiescence of the protrusive activity of the cell body’s lamellae. How actin polymerization and stability are balanced during the development of immature neurites, and its roles in asymmetrical growth of neurites into axons and dendrites is not well understood. Septins are a family of filamentous GTP-binding proteins, which associate with the cytoskeleton and membranes, and septin 7 (Sept7) has been shown to determine the sites of neurite formation in neuronal progenitors. Here, we have sought to investigate the role of septins in early neurite formation and growth. In embryonic (E18) rat hippocampal neurons, Sept7 localized to transverse arc filaments at the cortex of the cell body, where it colocalized with myosin IIA and B, and was also present at the base of cell body filopodia. Sept7 knock-down results in enlarged cell bodies, which lack pyramidal morphology and are characterized by extensive lamellipodia-like protrusions. Neurite number increased, and neurites extended directly from lamellipodial protrusions rather than from a consolidated cell body. In addition, individual neurites displayed enhanced protrusive activity, with increased branching compared to control. These phenotypes were accompanied by a reduction in the number of neurons that broke symmetry and developed a single elongated tau-positive neurite, the presumptive axon. Treatment of Sept7-depleted neurons with the Arp2/3 inhibitor CK666 or co-expression of dominant-negative cortactin rescued cell body shape and size, and neurites originating from a consolidated cell body, indicating that Sept7 suppresses Arp2/3-driven actin polymerization. Interestingly, while expression of the constitutively active myosin II regulatory light chain in Sept7-
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depleted cells did not rescue the enlarged cell body and neurite origin phenotype, it restored neurite number as well as axonal specification. Taken together, our data show that Sept7 is critical regulator of actin polymerization and contractility during neurite morphogenesis. We posit that Sept7 coordinates the actin dynamics of neurite formation by suppressing Arp2/3-based actin polymerization in the cell body and promoting myosin II contractility in newly formed neurites.

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**Branched Actin Regulated by CDC-42 Helps Nuclei Migrate Through Narrow Constrictions in C. elegans**

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Many developmental events require cells to migrate through constricted spaces. This process is often limited by the large and rigid nucleus. The mechanisms facilitating nuclear migration through narrow spaces are unclear. Using P cells in C. elegans, we can study nuclear migration in vivo. P-cell nuclear migration is facilitated by the linker of the nucleoskeleton and cytoskeleton (LINC) complex, which acts as a bridge, transferring forces generated by the cytoskeleton in the cytoplasm to structures inside the nucleus. Disruption of the LINC complex in P cells leads to a temperature-sensitive nuclear migration defect. At 25°C, less than 50% of P-cell nuclei successfully migrate to a ventral position, but at 15°C, at least 90% of P-cell nuclei successfully migrate. We therefore hypothesized that there is an additional nuclear migration mechanism pathway that functions in the absence of LINC to move nuclei. Using unbiased forward-genetic screens, our lab previously identified a role for toca-1 (transducer of CDC-42 activity) in P-cell nuclear migration. Here we report that our forward genetics screen also identified that a predicted CDC-42 guanine nucleotide exchange factor, CGEF-1, functions in the pathway. We hypothesized that a branched-actin pathway regulated by CDC-42 functions independently of LINC to squeeze P-cell nuclei through constricted spaces. In order to determine which CGEF-1 isoform is involved in this process, rescue constructs of CGEF-1a,c and CGEF-1b were expressed. Both constructs were able to rescue the nuclear migration defect, suggesting the short isoform of CGEF-1a,c is sufficient for P-cell nuclear migration. To test whether CDC-42 or the Arp2/3 complex were also required, we needed to use a tissue-specific knockdown since loss-of-function mutations in cdc-42 or the Arp2/3 component arx-3 are lethal. We used the auxin-inducible degradation system (AID) to specifically knockdown proteins during P-cell nuclear migration in the first larval stage. CRISPR/Cas9 editing was used to add sequences encoding an AID to cdc-42 and arx-3. We expressed the TIR ubiquitin ligase with a P-cell specific promoter. We then exposed animals to auxin a few hours before P-cell nuclear migration. Knockdown of either CDC-42 or ARX-3 led to a strong nuclear migration defect, but only in the absence of LINC complexes. We conclude that CDC-42 regulates a branched-actin network that functions during P-cell nuclear migration through constricted spaces. Our current model is CDC-42 is recruited to the nuclear envelope during P-cell nuclear migration where it is activated by CGEF-1 to initiate the formation of branched actin through TOCA-1 and the Arp2/3 complex.
**Actin regulation of oligodendrocyte morphology in the central nervous system**

**K. D. Dahl, W. B. Macklin; University of Colorado Anschutz Medical Campus, Aurora, CO.**

Myelin is a specialized membrane that ensheathes axons to increase the speed and efficiency of neuronal signal transmission. Deficiencies in myelin are associated with disease states such as multiple sclerosis that compromise movement and cognitive abilities. In the central nervous system myelin is produced by oligodendrocytes, which must undergo immense membrane growth and intricate morphology changes for normal function. Cytoskeleton dynamics are crucial for morphology changes, but it is not well understood how cytoskeleton dynamics are regulated in oligodendrocytes. mTOR signaling is important for increased filamentous actin in oligodendrocytes during early morphology changes but it has not been determined how mTOR functions in this way. I focus on mTOR complex 2 (mTORC2), as it has been shown to regulate actin dynamics in other cell types. mTORC2 can regulate Rac1 activation, which is necessary for early morphology changes in oligodendrocytes. Rac1 activation requires interaction with guanine nucleotide exchange factors, GEFs, at least one of which (TIAM1) has been identified to mediate Rac1 activation downstream of mTORC2. Thus I hypothesize that during early oligodendrocyte morphology changes, Rac1 activation is dependent on mTORC2 through the positive regulation of one or more GEFs. Exemplifying the importance of mTOR in cytoskeletal regulation during oligodendrocyte development, we see that pharmacological inhibition of mTOR (both mTORC1 and mTORC2) deceases both filamentous actin (F-actin) and active Rac1 in vitro. Additionally, knock out of the mTORC2-defining protein, RICTOR, reduces oligodendrocyte complexity in vivo and in vitro. This demonstrates that the loss of mTORC2 signaling negatively impacts oligodendrocyte complexity, possibly through decreased Rac1 activation and F-actin.

**Bulk actin dynamics drive phase segregation in zebrafish oocytes**

**S. Shamipour, R. Kardos, S. Xue, B. Hof, E. Hannezo, C. Heisenberg; Institute of Science and Technology, IST Austria, Klosterneuburg, AUSTRIA.**

Segregation of maternal determinants within the oocyte constitutes the first step in embryo patterning. In zebrafish oocytes, extensive ooplasmic streaming leads to the segregation of ooplasm from yolk granules along the animal-vegetal axis of the oocyte. We show that this process does not rely on cortical actin reorganization, as previously thought, but instead on a cell-cycle-dependent bulk actin polymerization wave traveling from the animal to the vegetal pole of the oocyte. This wave functions in segregation by both pulling ooplasm animally and pushing yolk granules vegetally. Using biophysical experimentation and theory, we show that ooplasm pulling is mediated by bulk actin network flows exerting friction forces on the ooplasm, while yolk granule pushing is achieved by a mechanism closely resembling actin comet formation on yolk granules. Our study defines a novel role of cell-cycle-controlled bulk actin polymerization waves in oocyte polarization via ooplasmic segregation.
**Targeted Destruction Of Muscle Myosin II In An Intermediate Step Of Myofibrillogenesis Is Essential For Formation Of Mature Myofibrils**

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Cross-striated muscle's repeating contractile units are arranged in sarcomeres that are linked end to end in myofibrils. Once organized in the sarcomeres, the arrays of force-generating proteins require continued maintenance as sarcomere numbers increase, and myofibrils extend in length and width. Understanding how the structural framework is assembled from sarcomeric proteins, and maintained to generate continual force provides the foundation for interpreting how myopathies occur in muscles.

Studies of de novo assembly of myofibrils in vertebrate cross-striated muscles have shown that myofibril assembly occurs in a three-step sequence that begins with premyofibrils, which progress to formation of nascent myofibrils, and concludes with mature myofibrils. Premyofibrils are arranged in minisarcomeres composed of bands of nonmuscle myosin II aligned along actin filaments that are anchored to muscle-specific alpha-actinin in Z-Bodies along the myofibril membranes. The incorporation of titin and muscle myosin II filaments in premyofibrils leads to the formation of nascent myofibrils with two different isoforms of myosin II: short filaments of nonmuscle myosin II and overlapping thick filaments of muscle myosin II. Mature myofibril formation is characterized by the absence of nonmuscle myosin II, and the addition of late-assembling proteins (telethonin, myomesin, and muscle myosin II binding protein C). Z-Bodies align into Z-Bands, and muscle myosin II filaments organize into A-Bands with titin stretching from Z-Bands to the middle of the A-Bands in mature myofibrils. Myofibrillogenesis can be halted at the transition from nascent myofibrils to mature myofibrils by three different modes of inhibition acting at specific sites in the Ubiquitin Proteasome System (UPS): (1) inhibition of ubiquitination of sarcomeric proteins by E3 Cullin ligases; (2) inhibition by p97 protein extraction of ubiquitinated sarcomeric proteins; and (3) inhibition of proteolysis by proteasomes. These three different types of UPS inhibitions indicate the importance of removing sarcomeric proteins from the nascent myofibrils to permit their transition to mature myofibrils. Liquid chromatography-mass spectroscopy analyses of proteins from control and UPS inhibitor-treated quail myotubes demonstrated that there is a large increase in the presence of ubiquitinated muscle myosin II heavy chains. In contrast to these UPS inhibitors, myofibrillogenesis was not affected by inhibitors of autophagy and/or lysosomes. Our experimental results with UPS inhibitions of myofibrillogenesis suggest a possible explanation for the reported off-target effects on cardiac and skeletal muscles with proteasomal inhibitors used for multiple myeloma patients.

**Cancer Therapy: Defining Therapeutic Targets and New Therapeutics 2**

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**Treatment with Foxy-5, a WNT5A-mimicking peptide, impaired the interplay between autophagy, ROS production, chemotaxis and proliferation in leukemia microenvironment**

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Mesenchymal stromal cells (MSC) are essential bone marrow (BM) microenvironment structures that interact with hematopoietic stem cells (HSC), display physical support and release soluble factors. In myeloid malignancies, as Myelodysplastic Syndrome and Acute Myeloid Leukemia (AML), abnormal alterations occur in the BM niche, such as modifications in HSC and MSC interaction, deficient secretion of growth factors or inhibitors. WNT5a is a glycoprotein secreted by MSC that activates the WNT non-canonical pathway in HSC and modulates biological processes. Underexpression of Wnt5a is associated with AML development, and correlates with poor prognosis. Hence, our aim was to investigate the effects of a new WNT5a-mimicking compound (Foxy-5, kindly provided by WNTResearch), in leukemia. A panel of leukemia cell lines (U937, HL60, THP1, KG1a, K562) were treated with Foxy-5 in a time and dose-manner, in order to restore WNT5a levels. Reactive oxygen production (ROS), autophagy, actin polymerization, and proliferation were analyzed by flow cytometry, using DCFHA dye, acridine orange staining, Phalloidin antibody, and cell counting respectively; chemotaxis was evaluated by Transwell assay. Foxy-5 reduced ROS production in U937 (fold-decrease [FD] of mean fluorescence intensity [MFI]: 46.6 and 48.2), HL60 (MFI: 10.5 and 47.1), THP1 (MFI: 19.4 and 20.6), KG1a (MFI: 16.3 and 34.9) and K562 (MFI: 0.1 and 19.0) at 50 and 100μM (all P<0.05). Then, we evaluated proliferation and migration, since these processes are modulated by ROS. Foxy-5 compound severely impaired CXCL12-induced chemotaxis in U937 (FD: 27.9), HL60 (FD: 42.5), THP1 (FD: 82.4) and K562 (FD: 45.1) at 100μM (all P<.05) probably by decreased cytoskeleton dynamics through lower actin polymerization rate in U937 (MFI: 30.8), THP1 (MFI: 29.1) and K562 (MFI: 23.9) at 100μM (all P<.05) and disruption onto PI3K and MAPK signaling activation. Besides, this compound probably acts as a sensitizing agent by decreasing ROS production and promoting reduced chemotaxis. Foxy-5 significantly reduced proliferation in U937 (FD: 41.0), HL60 (FD: 18.0), THP1 (FD: 36.0) and K562 (FD: 68.0) at 100μM (all P<.05). We also performed an adherent two-dimensional (2D) system with a direct crosstalk between mesenchymal HS5 cell and leukemia cells. Interestingly, coculture of leukemia cells in mesenchymal HS5 cells monolayer decreased cell autophagy, which was not observed in monoculture. In 2D culture, Foxy-5 reduced autophagy in U937 (MFI: 27.8), HL60 (MFI: 35.9), KG1a (MFI: 16.4) and K562 (MFI: 35.8) at 100μM (all P<.05). Autophagy is required for sustaining leukemia cell growth by induction of ROS production. Thus, the reduction of autophagic events in BM niche after Foxy-5 treatment may be a strategy to disrupt leukemia growth and maintenance.

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The Future of Anticancer Drugs: A Cytotoxicity Assessment Study of CdSe/ZnS and InP/ZnS Quantum Dots

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Quantum dots (QDs) are nanoparticles that emit various wavelengths of fluorescent light, allowing them to be exploited in vivo sensing/imaging of cancer cells. Cd based QDs have been a popular research choice among scientists, but concern for their cytotoxic properties have been expressed. InP QDs are hypothesized to be a less toxic alternative to Cd based QDs. Because of this, InP QDs are an emerging option in QD technologies for uses of fluorescent imaging as well as targeted drug and anticancer therapies based on their customizable properties. In the present study, CdS/ZnS and InP/ZnS QD were observed side by side in HeLa cervical cancer cells. Cell viability, reactive oxygen species (ROS),
apoptosis, and RNA-seq were investigated in both QD treatments. With CdS/ZnS treatment cell viability was decreased and late apoptosis was induced; ROS production was not significantly changed. RNA-seq revealed significant upregulation of antiapoptotic and antitumorigenic functions. In InP/ZnS treated cells, viability decreased, and levels of apoptosis were elevated. Differing from CdS/ZnS treatment, InP/ZnS treated cells observed production of superoxide molecules and nitrogen radicals. RNA-seq revealed processes involving proliferation to be downregulated, as well as apoptosis and metastatic inhibition to be upregulated. Collectively, the results of this project provide evidence that CdS/ZnS and InP/ZnS QDs can be considered for anticancer drug therapies. Our transcriptome analysis provides new insight in the alteration of physiological state of HeLa cells when introduced to Cd and InP QDs.

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Antiproliferative properties of ethanolic and aqueous graviola leaf extracts on tongue squamous cell carcinoma cell line-25

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Background: Annona muricata, commonly known as Graviola, soursop or guanabana, is an evergreen tree native to the tropics with a long history of use in ethnomedicine in indigenous communities in Africa and South America. Its active phytoconstituents have provided medicinal benefits against various ailments and diseases such as arthritis, parasitic infection, hypertension, fever, or diabetes. Studies conducted in vitro and in vivo have concluded that Graviola phytocomponents have anti-cancer and anti-tumor properties. One of the characteristics of cancer cells is their uncontrolled proliferation rate. In that sense, molecules that inhibit cell proliferation offer potential therapeutical benefits. Methods: We prepared ethanolic and aqueous extracts from dried Graviola leaves and tested their respective antiproliferative activities on tongue Squamous Cell Carcinoma cell line-25. We treated the cells with increasing concentrations of the extracts for 24 h. The respective doses leading to a 50% inhibition of cells growth (GI50) were determined. Results: Our results showed that the ethanolic extract was 4 times more active in inhibiting the growth of Squamous Cell Carcinoma cell line-25 than the aqueous extract (respective GI50 of 61.7 μg/mL, and 274.6 μg/mL). Conclusion: We hypothesize that some organic compounds involved in the antiproliferative/cytotoxicity of Graviola leaves were selectively extracted by Ethanol. Future plans include characterizing those bioactive compounds and assessing their activity on Squamous Cell Carcinoma cell line-25 vs. non-cancerous oral cells. Our hope is to discover natural molecules to be used as alternative treatments for oral Squamous Cell Carcinomas.

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Intein-mediated cytoplasmic reconstitution of a split toxin enables selective cell ablation in mixed populations and tumor xenografts

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The application of proteinaceous toxins for therapeutic and experimental cell ablation is limited by their high on- and off-target toxicity leading to severe side effects and a narrow therapeutic window. The selectivity of targeting can be improved by splitting a toxin into two dysfunctional benign fragments and delivering them to the cytoplasm of the target cell via independent selective pathways. The reconstitution of a functional toxin in the cytoplasm of the target cell can be achieved via trans-splicing
by split inteins, an indispensable tool in protein engineering, biotechnology, and experimental therapeutics. While intein-mediated reconstitution of proteins from genetically encoded elements has been explored, exploiting cell surface receptors for intracellular delivery of intein-flanked protein fragments to boost selectivity has not been achieved. The goal of the current study was to expand the applicability of the split-intein technology by enabling selective, receptor-mediated delivery of split-intein based protein constructs across biological membranes for cytoplasmic trans-splicing in live-cell and in vivo applications. We designed a robust splitting algorithm and achieved reliable intracellular reconstitution of functional Diphtheria toxin from engineered intein-flanked fragments upon transmembrane receptor-mediated delivery of one of them to the cells expressing the counterpart. Retargeting the delivery machinery towards different receptors overexpressed in cancer cells enabled selective ablation of specific sub-populations in mixed cell cultures. In a mouse model, the transmembrane delivery of a split-toxin construct potently inhibited the growth of subcutaneous xenograft tumors expressing the split counterpart. Receptor-mediated delivery of engineered split proteins provides a platform for precise therapeutic and experimental ablation of tumors or desired cell populations while also greatly expanding the applicability of the intein-based protein trans-splicing.

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Antimicrobial Activities and DNA Interactions of (N/N) Cyclotriphosphazenes with 4-Methoxybenzyl Pendant Arm

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In the last two decades, some of the cyclophosphazene derivatives have been used in pharmaceutical areas. Due to their importance in cancer treatment and their biomedical applications attracting great attention, this situation creates more research in this field. Aminocyclotriphosphazenes exhibit cytotoxic activity against HT-29 (human colon adenocarcinoma), Hep-2 (human epidermoid larynx carcinoma) cells and induced apoptosis in HeLa cancer cell lines [1-2]. In this study; first of all, partially and fully substituted mono-4-methoxybenzylaminophosphazenes were synthesized by Cl replacement reactions. For this, the reactions of 4-methoxybenzyldiamines with hexachlorocyclotriphosphazene (trimer; N3P3Cl6) gave the new monospirocyclotriphosphazenes with the 4-methoxybenzyl (anisole) pendant arm. The tetra-pyrrolidino and tetra-piperidino-substituted monospiro compounds were prepared from the reactions of tetrachloro derivatives with excess pyrrolidine and piperidine. Later, with the application of agar well diffusion method, it was observed that some new cyclotriphosphazenes have antimicrobial activity against Gram (+) and Gram (-) bacteria and also have antifungal activity against yeast strains. Moreover, MIC, MBC and MFC values of these phosphazenes were also determined. On the other hand, interactions between pBR322 plasmid DNA (intracellular target) and synthesized phosphazenes were investigated by agarose gel electrophoresis. To find changes in DNA confirmation, compound-DNA incubation is restricted with BamH1/HindIII enzyme digestion. According to the results, it is observed that compounds with different structural properties exhibit different DNA cleavage and binding capacities and the compounds can cause a small conformational change in DNA. References;[1] T. Yildirim, K. Bilgin, G. Yenilmez Çiftçi, E. Tanrverdi Çek, E. Şenkuytu, Y. Uludağ, L. Tomak and A. Kilic, Eur. J. Med.Chem., 2012, 52, 213-220.[2] N. Asmafiliz, Z. Kilic, T. Hökelek, L. Acik, L. Y. Koc, Y. Süzên and Y. Öner, Inorg. Chim. Acta, 2013, 400, 250-261.
Photodynamic therapy using chloro aluminum phthalocyanine and doxorubicin combined nanoemulsion leads diminution of cellular proliferation, vascular damage and in vivo necrosis

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Background: Breast cancer is a public health problem due to high incidence, mortality and ineffective treatment, which leads to the search for new treatments. This study aimed to investigate nanoemulsions activity (chloro-aluminum phthalocyanine and doxorubicin – NE-PcDoxo) associated with photodynamic therapy (PDT) in vivo.

Methods: Forty female BALB/c mice received different treatments as briefly described: 1 - NE-Doxo administration without PDT; 2 - NE-PcDoxo administration associated with PDT; 3 - NE-Pc administration associated with PDT and after 24 hours NE-Doxo administration (adjuvant treatment); 4 – animals without any treatment (Control); 5 - NE-PcDoxo administration without PDT; 6 - NE-Pc administration associated with PDT; 7 - treated with laser irradiation; 8 - NE-Pc administration and after 24 hours NE-Doxo administration (adjuvant treatment) without PDT. The animals were divided into 8 groups (n=5) and 2x10^5 4T1 cells diluted in 100µl of phosphate-saline buffer were inoculated in the fourth right mammary gland, aiming the tumor development at the injection site. After nanoemulsion administration (intratumoral injections of 100µl nanoemulsions - 1µM of chloroaluminum phthalocyanine associated/or 0.5µM of doxorubicin diluted in 0.9% NaCl) were administered. Laser irradiation at 630nm of 50 J.cm^{-2} was applied every 24 hours during three days and finally, animals were euthanized after the last PDT session and the tumors were collected for analysis. Immunohistochemical and Western Blotting analysis was performed for Ki67, PCNA and CD31 antibodies. Slides were scanned and images obtained (10x magnification) followed for stereological analysis using M130 multipoint system (Image-Pro Plus grid mask tool). Five cross-sections from the central and peripheral regions of the tumors were stained with H&E and the percentage index of proliferation, initial necrosis and advanced necrosis were noted.

Results: Mice treated with NE-PcDoxo and PDT presented a significant decrease of proliferation cells compared to other evaluated groups, as well as the level of PCNA and CD31 protein expressed in that group was significantly decreased. The characterization of tumor areas by stereological analysis demonstrated that there was a significant decrease of proliferative tumor area. In contrast, areas of initial and advanced necrosis were increased in the proposed treatment. Conclusion: NE-PcDoxo formulations associated with PDT were efficient in breast tumor reduction and promoted tissue necrosis. It was possible to verify positive responses with this treatment and these promising results may allow a new therapeutic approach to breast cancer. Approval of Committee on Ethics in the Use of Animals (protocol nº 081/2013 IBILCE/UNESP). Financial Support: FAPESP.
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_Meso-tetra (4-carboxyphenyl) porphyrin (TCPP) is incorporated into cancer cells by the CD320 receptor and clathrin mediated endocytosis_

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Porphyrs have been used successfully to treat and diagnose cancer, yet the mechanism of how porphyrins are selectively taken up and how they are retained by cancer cells compared to other cells remains poorly understood. Knowledge about the cellular uptake and retention mechanisms of porphyrins can be used to design more effective porphyrin-based diagnostics and therapeutics. We designed a flow cytometry assay to measure the cellular incorporation of meso-tetra (4-carboxyphenyl) porphyrin (TCPP) into cancer cell lines. We choose to study TCPP because it is currently used in a diagnostic assay for the early detection of lung cancer. By using inhibitors specific to different endocytosis pathways, we have shown that TCPP enters cancer cells by a clathrin-mediated-endocytosis pathway. Cell-based ELISA assays have been performed to verify the specificity of the endocytosis inhibitors used. The LDL receptor, previously implicated in porphyrin uptake, only contributed modestly to TCPP uptake. In search of alternative TCPP entry mechanisms, we found that CD320, the cellular receptor for cobalamin/transcobalamin II (Cbl/TCN2), bound strongly to TCPP (Kd = 42 nM). Short hairpin RNA-mediated knockdown of CD320 resulted in up to 40% decrease in TCPP uptake in cell lines derived from lung, breast, and prostate cancer. The sub-cellular localization of CD320 was altered upon TCPP exposure, as measured by immunofluorescence microscopy. Our results as a whole support our hypothesis that TCPP binds to CD320 and uses this receptor to enter cancer cells. Given that cancer cells over express CD320, our research provides a reason for why TCPP accumulates in cancer cells.

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_Targeted Delivery of FADD Protein in Cancer Cells, Dually Regulates Apoptosis and NF-κB Signaling_

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Fas Associated Death Domain (FADD) protein is central to implicate cell death regulatory pathways, however, a heterogeneous expression and post-translational modifications of FADD in cancer cells, leads to impediment in their functional outcomes. This intricacy is further buttressed by the fact that, a constitutive NF-κB activity in cancer cells, usually antagonizes FADD mediated cell death pathways, through upregulation of anti-apoptotic machinery. In light of this, very few anti-cancer molecules have been clinically epitomize, showed dual regulation of apoptosis and NF-κB signaling. We and others have previously shown that, the induced expression of FADD in cancer cells, ameliorates apoptosis with concomitant suppression of NF-κB dependent downstream signaling. Further, we focused to develop FADD as a protein therapy, and investigated their competency to regulate apoptosis and NF-κB signaling. To achieve this, we used TAT cell penetrating peptide, and conjugated them with purified FADD protein, for efficient intracellular delivery. The biological efficacy of TAT-FADD was examined in vitro and in vivo tumor model. We found that, 5µM of TAT-FADD within 3h of treatment, endocytosed through membrane caveolin and, assembled death inducing signaling complex (DISC) in HCT116, HeLa and MCF-7.
cancer cells. Moreover, the viability of cancer cells, including HCT116, HeLa, MCF-7, and HepG2 and, transformed cells HEK293 and RAW264.7 were reduced to 50% in 12h of TAT-FADD treatment as compared to untreated cells. Importantly, around 45% of apoptotic death achieved within 12h of TAT-FADD protein treatment, as compared to similar percentage observed with 48h of FADD transfected cells. Mechanistically, TAT-FADD regulates TNFα induced NF-κB activation (6 fold), and induces ubiquitination of TRAF2 signaling complex to impede NF-κB signaling. In addition, TAT-FADD suppresses NF-κB downstream anti-apoptotic genes, including, Bcl2 (3 fold), cFLIP, (5 fold), cIAP2 (2.5 fold) and RIP1 (3 fold), independent of TNFα priming. In addition, TAT-FADD targets the NF-xB mediated pro-inflammatory activation of NLRP3 inflammasome complex subunits, including NLRP3, procaspase-1 and pro-IL1β, both at the transcriptional and translational levels. We further validated our in vitro outcomes in an in vivo tumor model. The administration of TAT-FADD (50 mg/kg), post 3 weeks of treatment reduces around 2.8 fold tumor volume with concurrent activation of pro-apoptotic caspase-8 and -3. The TAT-FADD treated group showed a reduced p65 expression and IL1β levels in tumor cells. Altogether, we report prospective, therapeutic relevance of FADD protein in dual regulation of apoptosis and NF-κB signaling in cancer cells.

Cell Adhesion Signaling in Migration

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Trop-2 inactivates E-cadherin and drives metastasis in most aggressive cancer types
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Metastatic diffusion requires loss of cell-cell adhesion. We discover here that functional inactivation of E-cadherin by Trop-2 drives cell-cell detachment and underpins metastasis in high-incidence cancer types. Gene expression profiling identified TROP2 as a unique up-regulated gene in metastasis. Trop-2 overexpression induced E-cadherin-cytoskeleton uncoupling, cell-cell junction ablation, and activation of β-catenin and downstream transcriptional targets. We showed that this mechanism leads metastatic diffusion in vivo. An E-cadherin-inactivation metastasis program was then found recapitulated in breast, colon, uterus, ovary, stomach, lung, pancreas cancer patients, over 24 independent case series, encompassing 13,042 primary tumours. Aggressive tumours, such as triple-negative breast cancers, were driven toward global relapse by E-cadherin inactivation, whereas no disease recurrence was observed in control cases. These finding lead to the novel paradigm of a Trop-2-driven, E-cadherin-inactivation program as metastasis driver across cancer types. This may open far-reaching perspectives in diagnostic procedures and anti-cancer therapies.

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Differential adhesome expression defines human natural killer cell residency and developmental stage
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Human natural killer (NK) cells are large granular innate immune cells that circulate in peripheral blood and mediate cellular cytotoxicity against virally infected and tumorigenic target cells, primarily in tissue microenvironments. Human NK cell development is the progressive maturation of CD34⁺ progenitors into terminally mature (CD56dim) NK cells and can be defined by the acquisition or loss of cell surface receptors and intracellular components which define unique phenotypic developmental stages. Previous studies defining the physiological distribution of NK cells, together with those that demonstrate NK cell interactions with developmentally supportive stromal cells, serve as evidence that NK cells must be able to interact with a variety of complex microenvironments throughout their differentiation and as mature functional cells. The integrin adhesome represents a molecular network of integrin mediated signaling interactions that mediate cell behavior, development, migration, and localization. In this study we aimed to define the integrin adhesome profile of NK cell developmental subsets from tonsil tissue, a documented site of NK cell differentiation and maturation. These cells were compared with mature NK cells from peripheral blood and NK cells generated from hematopoietic precursors through stromal cell co-culture. We hypothesized that both tissue residency and developmental stage would drive differences in adhesome gene and protein expression between NK cell populations. Phenotypic analysis by bulk RNA-seq and flow cytometry revealed that NK cell subsets have distinct expression of adhesome associated genes and proteins, including differential regulation of integrins, actin regulators and cytoskeletal components that are driven by both NK cell developmental stage and site of tissue residency. Further, we found greater cortical actin density in terminally mature NK cells, particularly those isolated from peripheral blood. This increased actin density, detected by microscopy and flow cytometry, was accompanied by higher expression of actin nucleation regulators including Arp2 and profilin. This observation links understanding of differential integrin-mediated actin signaling networks with observed differences in cortical actin content in human NK cells. Overall, our study provides a new understanding into the diversity of human NK cell populations and how they interact with their microenvironment.

Crosstalk between Focal Adhesion Kinase/Src and Protein Kinase A signaling pathways in cellular migration

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Directed cell migration requires cells to sense the extracellular environment and integrate extracellular cues into directional movement. Just as cells respond to extracellular chemical gradients, they respond to changes in the mechanical properties of the microenvironment. Durotaxis is a process by which cells sense and respond to gradients in extracellular matrix stiffness, seeking out stiffer regions of a tissue. These processes require the coordination of myriad intracellular signaling molecules in time and space. Previously, using a FRET-based biosensor, we have shown that the cAMP dependent protein kinase (PKA) is active at the leading edge of randomly migrating SKOV-3 ovarian cancer cells and that this activity is dependent on cellular contractility. We also demonstrated that PKA activity is required for durotaxis and that PKA activity can be potentiated by durotactic stretch. Among many other signaling pathways important for cell migration, Focal Adhesion Kinase (FAK) and Src family kinases have been established as key regulators of this process. Though there have been few reports of FAK/Src signaling pathways acting upstream of PKA, we have observed that leading edge PKA activity is dependent on FAK/Src signaling. Further, FAK has established roles in mechanical signaling pathways and durotaxis.
Using pharmacologic and genetic manipulation, we investigate the intersection of the canonical pathway of PKA activation, via G proteins and adenyl cyclase, and novel inputs from tyrosine kinases FAK and Src upstream of this activity in both random cell migration and in the durotactic response.

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Ste20-like kinase SLK regulates collective cell migration through redistributing E-cadherin in oral cancer cells

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Collective cell migration is defined as the coordinated movement of multiple cells that retain cell-cell contacts while coordinating their actin dynamics and intracellular signaling. Such coordination of cell groups is a key event in organogenesis and in disease states such as cancer metastasis. During collective cell migration, cells remain cohesive by expressing cell-cell junction molecules such as cadherins or adhesion receptors of the immunoglobulin superfamily. Here we identified Ste20-like serine-threonine kinase (SLK) as a pivotal role in collective cell migration. Using oral cancer cell line Cal27 as experimental material, we found that SLK knockdown significantly improved cell-cell coordination, but the sheet migration speed was not altered by SLK manipulation. Metastasis have been proposed to initiate following loss of the intercellular adhesion protein, E-cadherin, on the basis of inverse correlations between in vitro migration and E-cadherin levels. However, the latest research reveals that E-cadherin may enhance metastatic spread and outgrowth of cancer cells. We find that SLK knockdown may increase E-cadherin expression on cell membrane, thus accelerate cell-cell adhesion. Furthermore, we find that knockdown of SLK redistribute E-cadherin via regulating its trafficking process in OSCC cells, instead of the expression levels.

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A Genetic Suppressor Screen to Find Novel Regulators of Tumor Suppressor Homolog Kinase Responsive to Stress B (KrsB)

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*Dictyostelium discoideum* social amoeba is a well-established model organism for the study of amoeboid-type migration, which is the type of movement seen in neutrophils and metastatic cancer cells. Cycling between active and inactive forms of the serine/threonine kinase responsive to stress B (KrsB), a homolog of mammalian tumor suppressor MST1/2 and Drosophila Hippo, contributes to the dynamic regulation of cell adhesion that is needed for proper cell adhesion and chemotaxis in *D. discoideum*. However, the exact mechanism by which KrsB affects the cell’s ability to adhere is unclear. The goal of this project is to find new regulators or effectors of KrsB using a genetic suppressor screen. Cells lacking KrsB have a distinct phenotype when they form plaques on a bacterial lawn, with an enlarged region of cells in streams and an uneven expanding front of vegetative cells, which makes *krsB* plaques appear to have rough edges. Cells lacking KrsB were transformed with a cDNA library. To enrich the population of rescue cells following antibiotic selection, plates were placed on a rotational shaker.
for one hour, after which floating and adherent cells were plated on bacterial lawns separately. 29 plaques exhibited either a rescue or an enhancement of the original phenotype, with 21 plaques originating from the floating cell fraction and 8 from the adherent cell fraction. 22 of the plaques had rescue-like morphology with smooth round edges or more developed fruiting bodies than KrsB-null plaques. The rest of the plaques looked much smaller and underdeveloped in comparison to wild-type or KrsB-null plaques. All of the mutants were collected, grown, and subjected to a secondary screen by clonally replating them on bacterial lawns. Five mutants exhibited a rescue-like phenotype and five exhibited a more severe one, all consistent with the identified plaque morphology in the initial screen. Identification of the cDNA inserts in these mutants is currently ongoing. Determining the genes responsible for the rescue of the krsB phenotype or for making the phenotype more severe will give us a better understanding of the molecular mechanism of KrsB function in cell adhesion and migration.

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Physiological role of CMG2 in intestinal lymphatic development

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Loss-of-function mutations in the capillary morphogenesis gene 2 (CMG2) cause hyaline fibromatosis syndrome (HFS), a rare genetic disorder characterized by the growth of subcutaneous nodules, gingival hypertrophy, and joint contractures, with severe forms leading to intestinal disorders and death. However, the physiological role that CMG2 and its mutation play in this disease and its progression are currently unknown. Given that the HFS intestinal phenotype is associated with intestinal lymphangiectasia and is generally lethal, our goal was to investigate the role of CMG2 in intestinal lymphatic system development. Using whole mount microscopy on mesentery of CMG2 +/+ and CMG2 -/- in newborn mice, we demonstrated that CMG2 knockout mice show decreased lymphatic vessel branching, decreased number of lymphatic valves in pre-collecting vessels, and valve maturation defaults in collector lymphatic vessels. In addition, lymphatic vessel diameter was increased in mesentery whereas blood vessels diameter was not affected by CMG2 deletion. Similarly, intestinal lacteals of adult CMG2 -/- mice were shorter and dilated in comparison to CMG2 +/+ mice. Because cell proliferation and migration are essential mechanisms for lymphatic network development, we used lymphatic endothelial cells treated or not with siRNA targeting CMG2 to investigate cell proliferation and cell migration assays. We show that upon CMG2 knockdown, cell proliferation and migration, through Boyden chamber assay and wound healing assay, are inhibited. Altogether these results show a specific role of CMG2 in lymphatic cells proliferation, migration and development of lymphatic network.

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A Feedback Loop between Adhesion Regulators Rap1 and Kinase Responsive to Stress B in Dictyostelium discoideum


Cell adhesion to the substrate influences a variety of cell behaviors and its proper regulation is essential for migration. Social amoeba Dictyostelium discoideum is a commonly used model organism, whose movement is very similar to that of other amoeboid cells, such as neutrophils and metastatic cancer
cells. Although we know many components of the signal transduction network that regulate directed migration, details of the pathways regulating cell adhesion during migration are lacking. Rap1 is a small GTPase that regulates adhesion in *Dictyostelium* cells in part via its effects on myosin II and talin. Kinase responsive to stress B (KrsB), a homolog of mammalian tumor suppressor MST1/2 and *Drosophila* Hippo, also regulates cell adhesion and migration, although the molecular mechanism of KrsB action is not understood. Since KrsB has been shown to interact with active Rap1 by mass spectroscopy, we decided to investigate the genetic interaction between Rap1 and KrsB. Cells lacking KrsB have increased contact with the substrate and are difficult to detach from the surface, which leads to reduced movement. Expression of constitutively active Rap1G12V increased cell adhesion, and inactive Rap1S17N reduced cell adhesion even in the absence of KrsB, suggesting that Rap1 does not require KrsB to mediate cell adhesion. However, Rap1G12V did not increase cell spreading in the presence of a high amount of KrsB and Rap1-mediated increase in spreading was dramatically enhanced in the absence of KrsB, suggesting that KrsB might have the ability to negatively regulate Rap1. In addition, chemoattractant-induced activation of Rap1, as assessed by transient cortical localization of the biosensor RalGDS, was impaired in krsB− cells, possibly due to increased basal activity of Rap1. Furthermore, Rap1G12V reduced cAMP-induced KrsB phosphorylation, whereas expression of Rap1S17N raised basal KrsB phosphorylation, suggesting Rap1 also regulates KrsB activation. Thus, Rap1 appears to activate KrsB, which may function in a negative feedback loop to shut off Rap1 signaling, allowing for precise regulation of cell adhesion during migration.

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**Single Molecular Force across Integrin αvβ3 Directs Cell Migration**  
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Cells sense their micro-environment and alter their morphology dynamically to adapt to continuously changing physical stimuli. Cell migration is one of the most essential features of diverse cellular functions such as wound healing, immune response, and cancer metastasis. Previous studies have shown that cell adhesion is determined by the integrin-mediated single molecular force. However, how the subcellular forces between cell and extracellular matrix determine cell migration remains unclear. Here we present that the single molecular force across integrin determines the cell motility by regulating integrin expression and molecular force-dependent protein activation. We control the integrin-mediated single molecular force precisely using double-strand DNA rupture force. Our results show that cell spreading area and focal adhesion size decrease as the integrin-mediated single molecular force becomes weak. Ultimately, adhesion-dependent cells display distinct migration modes in response to integrin expression and activation. These results demonstrate that adherent cells express enhanced integrin expression to overcome reduced adhesion force between a cell and extracellular matrix, which could maintain mechanical homeostasis of cells. Furthermore, we note that integrin activation requires a threshold value of the single molecular force between integrin and extracellular matrix. By combining subcellular cell mechanics and single molecular force measurement, we expect our results could provide a new insight onto the cell adhesion and migration in diverse in tissue environment.
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**GSK3β controls T-cell migration through Notch-GSK3β-CRMP2 regulatory axis**

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**Objective:** The aim of this study was to investigate the role of GSK3β in the regulation of T-cell migration. **Methodologies:** *In vitro* T-lymphocyte migration model, real-time chemotaxis, high content analysis, confocal imaging, Western immunoblotting, co-immunoprecipitation, mass spectrometry and Ingenuity Pathway Analysis (IPA\(^*\)). **Results and Discussion:** Migration-triggered signalling in human T-cells stimulated via interactions between the integrin LFA-1 and its ligand ICAM-1 augments GSK3β phosphorylation (Ser9, ≥2-fold) and its nuclear translocation. This process is mediated by Notch1-intracellular-domain (NICD), a critical rate limiting step, wherein inhibition of Notch activity abrogates both phoso-GSK3β(Ser9) and T-cell motility. Proteomics and protein network pathway analysis reveal a crucial association between GSK3β and collapsin response mediator protein (CRMP2) in motile T-lymphocytes. LFA-1/ICAM-1 stimulation, reduces CRMP2 phosphorylation (Thr514) and mitigates CRMP2 interaction with de-tyrosinated form of α-tubulin in migrating T-lymphocytes (< 2-fold). Moreover, specific inhibition of GSK3β activity by CHIR-99021, decreases phoso-CRMP2 (Thr514) and enhances the migratory potential of human T-cells. **Conclusion:** Phosphorylation of GSK3β(Ser9) and its nuclear translocation facilitated by NICD is a critical step in the multi-tier regulatory Notch-GSK3β-CRMP2 axis in T-cell migration.

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**Deciphering the roles of discoidin proteins in controlling cell mechanics**

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Processes such as cytokinesis, migration, and chemotaxis require robust and constant cell shape modifications. To complete such modifications, cells need to integrate internal and external cues through its contractility machinery, which includes actin filaments, myosin II motors, actin crosslinkers, such as Cortexillin I, and scaffolding proteins, *e.g.* IQGAP2. These proteins constitute a mechanosensory system that allows cells to receive as well as respond to chemical and mechanical signals through accumulation in order to direct cell shape changes. Previously, using a proteomics approach, we identified interactors of Cortexillin I and IQGAP2, two key nodes of the mechanoresponsive contractility machinery. Through this effort, we uncovered a broad network of biochemical interactors that potentially contribute to the activity and integrity of the system. Among these, discoidin proteins I and II emerged. Discoidin proteins are sugar binding proteins that contain a lectin and a discoidin domain. Though several previous studies suggested extracellular functions for discoidin in cell adhesion and multi-cellular development, the exact functions of discoidin remain elusive. Previously, we found that discoidin I interacts biochemically with myosin II and cortexillin I *in vivo*. Using CRISPR-Cas9, I generated knockouts of *discoidin* and found that these cells display a relatively mild cytokinesis defect. Strikingly, double mutants of *discoidin I* and *cortexillin I* have a less severe growth phenotype than the *cortexillin I* single null mutant cells, implicating a complex genetic interaction between these two proteins. These observations suggest that discoidin I and cortexillin I each have roles in cytokinesis, but discoidin I and
cortexillin I also have an antagonistic relationship. Future studies will include a complete detailing of the interactions between discoidin I and cortexillin I as well as the implications on the integrity and function of the mechanoresponsive contractile machinery.

Cell-Cell Junctions

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The depletion of the Coxsackie and Adenovirus Receptor (CAR) in the respiratory epithelium decreases inflammation but increases airway remodeling in a model of allergic airway inflammation

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Airway inflammation and remodeling are key pathophysiologic features process in many respiratory conditions such as asthma or COPD. An intact epithelial cell layer is crucial to maintain lung homeostasis, and this depends on intercellular adhesion, regulated by tight junctions and adherens junctions. The Coxsackie virus and adenovirus receptor (CAR) is a member of the immunoglobulin superfamily and functions as a cell-cell adhesion molecule through homophillic interaction \textit{in trans} to stabilise epithelial cell-cell adhesions. CAR is also a receptor for immune cells and facilitates transepithelial migration (TEpM) after inflammation in the skin and gut. We have previously demonstrated that CAR is regulated through phosphorylation to control junction stability in lung epithelial cells and TEpM of monocytes and neutrophils \textit{in vitro} and in acute respiratory inflammation \textit{in vivo}. Here we have investigated the mechanistic role of CAR in mediating responses to the common aeroallergen House dust mite (HDM). We demonstrate that \textit{in vitro} HDM treatment of human bronchial epithelial cells leads to destabilisation of cell-cell adhesions, increased cell permeability and that these phenotypes require HDM-dependent CAR phosphorylation. Administration of HDM over 5 weeks in mice lacking CAR in the respiratory epithelium leads to loss of peribronchial inflammatory cell infiltration, reduced goblet-cells, fewer neutrophils and γδT cells in the lungs and decreased IL-4 and IL-13 release. Further analysis revealed the release of HDM-dependent inflammatory cytokines by the epithelium is regulated by CAR, and that depletion of CAR reduces collagen I, fibronectin and a-SMA deposition in the lungs following HDM insult. Moreover, removal of CAR leads to increased contractility of the airways in ex-vivo lung slices, and CAR-dependent secretion from the epithelium results in enhanced primary human airway smooth muscle cell proliferation. Our data demonstrates that CAR is a novel central co-ordinator of lung inflammation through a dual role in decreasing leukocyte recruitment and subsequent tissue remodelling and may represent a strong target for future therapeutic development.

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Lipopolysaccharide (LPS) modulates gene expression in the blood-brain barrier

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Chronic inflammation is a hallmark of many neurodegenerative disorders. Although the central nervous system (CNS) can stave peripheral pathogens from crossing the blood-brain barrier (BBB) through a network of continuous endothelia, astrocytes, and pericytes, prolonged exposure to a pathogen can comprise this barrier. Basigin, a cell adhesion molecule, is found on the surface of endothelial cells and is thought to interact with Toll-like receptor 4 (TLR4). TLR4 recognizes lipopolysaccharide (LPS), found on
the outer membrane of Gram-negative bacteria. The activation of TLR4 produces pro-inflammatory cytokines, like interleukin-6 (IL-6). The purpose of the present study was to evaluate the expression pattern of Basigin and TLR4 in brain tissue stimulated with LPS for various times to mirror acute and chronic inflammation, as well as at different life stages to determine whether the expression pattern is dynamic. Isolated brain tissue from mice at postnatal day (P) 7, 30 and 180 were incubated in cell culture medium ± LPS for 3, 6, 12, or 24 hrs. Total protein was purified from the isolated tissue and used in an enzyme-linked immunosorbent assay (ELISA) to determine Basigin protein concentration. In addition, Basigin, TLR4, and IL-6 gene expression was localized in brain tissue via immunohistochemistry. The results of the study indicate that Basigin expression was significantly reduced in P7 brains in response to LPS treatment for 24 hours, when compared to control-treated age-matched brains. Conversely, Basigin expression was significantly increased in P180 brains in response to LPS treatment for 24 hours, when compared to control-treated age-matched brains. No significant change in Basigin expression was observed in P30 mouse brains in response to LPS treatment. Similarly, no significant change in TLR4 or IL-6 expression was observed at any age or treatment time tested. The differential expression of Basigin in P7 and P180 but not P30 animals suggests pathogenic influence is more likely in neonatal and adult, but not adolescent mice. The pattern of TLR4 expression did not mirror that of Basigin gene products, indicating that the role of Basigin on endothelial cells may not be to associate with TLR4, but perhaps to associate with other pro-inflammatory proteins, or act in its role as an inducer of matrix metalloproteinases.

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Mechanoregulation of the Adherens Junction-associated RNAi machinery through cross-talk with the Extracellular Matrix

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Loss of epithelial tissue integrity is widely observed in colon cancer, which is the third most common and second deadliest type of cancer. The Adherens Junction (AJ) is a mechanosensitive adhesion complex, essential for maintenance of epithelial tissue integrity, composed of E-cadherin and the catenin family of proteins. We have shown that the E-cadherin - p120 catenin partner PLEKHA7 is critical for epithelial integrity. We have also found that PLEKHA7 recruits the core components of the RNAi machinery, includingAGO2, DGCR8 and DROSHA, at mature apical AJs, to regulate miRNA levels and activity. Loss of PLEKHA7 results in disruption of junctional localization of the RNAi machinery, in miRNA dysregulation, in oncogene upregulation, and in pro-tumorigenic colon cell behavior, in vitro and in vivo. A common precursor to colon cancer that negatively influences epithelial integrity, is the abnormal physical stress exerted by extensive extra cellular matrix (ECM) remodeling during fibrotic conditions. We hypothesize that physical stress from the ECM results in disruption and loss of function of the AJ-associated RNAi machinery, predisposing cells to pro-tumorigenic cell behavior. To test our hypothesis, we have manufactured and extensively tested for reproducibility a 3-D printed cell stretcher, designed specifically to apply strain on epithelial cell monolayers. Using this device, as well as standard acrylamide-based cell stiffness assays, we applied different conditions of strain and stiffness to confluent Caco2 cell monolayers. Interestingly, localization of PLEKHA7 and of RNAi machinery components at AJs
was severely disrupted when we applied strain (2-2.5%) and stiffness (23-40kPa) conditions that are physiologically relevant to fibrotic conditions in the colon. Furthermore, western blot analysis of cells under high stiffness conditions revealed increased Src activity, which opposes localization of PLEKHA7 and RNAi components to AJs, pointing towards a mechanism of action. Interestingly, PLEKHA7 depletion in Caco2 cells also resulted in elevated levels of a series of master ECM remodeling regulators that are predicted to be targeted by PLEKHA7-associated miRNAs and are elevated in colon tumors. This result further revealed an extensive, bi-directional cross-talk between PLEKHA7 and the ECM. Together, our data point towards a novel mechano-sensitive mechanism tethering ECM remodeling with cell behavior, through an AJ-associated RNAi machinery.

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**PLEKHA5 and PLEKHA6 are PDZD11 binding and WW-domain containing PLEKHA proteins localized at cadherin-based junctions**

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PDZD11 (*PDZ domain-containing protein 11*) is a key interactor of PLEKHA7 (*Pleckstrin homology domain-containing family A member 7*), a junctional protein that is implicated in hypertension, glaucoma and susceptibility to staphylococcal α-toxin. PDZD11 localizes at adherens junctions in a PLEKHA7-dependent manner, where it stabilizes nectins to induce efficient junction assembly. PDZD11 also promotes the interaction of the transmembrane protein Tetraspanin-33 (Tspan33) with the N-terminal WW domains of PLEKHA7, thus allowing the docking of Tspan33 and of the α-toxin receptor ADAM10 to junctions. We report here the identification of PLEKHA5 and PLEKHA6, two additional pleckstrin homology (PH) domain-containing proteins, as new interactors of PDZD11. They share several structural characteristics with PLEKHA7, including the two N-terminal WW domains which directly bind PDZD11. PLEKHA5 and PLEKHA6 are present at epithelial and endothelial cadherin-based junctions, with a tissue distribution and a subcellular localization distinct from that of PLEKHA7. Although PLEKHA5 and PLEKHA6 are detected at apical junctions, as PLEKHA7, they are also detected along lateral contacts, in a pattern similar to E-cadherin. In addition, PLEKHA5 decorates microtubules in a PDZD11-dependent manner. We also provide evidence that PLEKHA5, PLEKHA6, and PLEKHA7 undergo liquid-liquid phase separation to form biomolecular condensates. Their junctional recruitment depends on different structural domains, the PH domain being essential for PLEKHA5 to go to junctions, while not for PLEKHA6 nor PLEKHA7. Moreover, these three proteins homo- and hetero-oligomerize, and mutually cooperate to promote their junctional localization. PLEKHA7 and PDZD11 stabilize PLEKHA5 and PLEKHA6 protein expression: both regulate PLEKHA5 protein but not mRNA level, by preventing a proteasome-dependent degradation in the case of PLEKHA7; both balance PLEKHA6 mRNA level, but only PDZD11 prevents a proteasome-dependent degradation of the protein. The role of PLEKHA5 and PLEKHA6 in cell-cell junctions structure and composition appears distinct from that of PLEKHA7, as their deletion does not result in similar phenotypes. Finally, PLEKHA5, PLEKHA6 and PLEKHA7 define different cellular pools of PDZD11, i.e. at the zonular junctions, at lateral contacts and along microtubules. PLEKHA7 brings PDZD11 to the zonular belt exclusively, while PLEKHA5 and PLEKHA6 also recruit it to lateral junctions. However, only PLEKHA6 rescues the junctional localization of PDZD11 in PLEKHA7 KO cells. On its side, PLEKHA5 brings PDZD11 to microtubules too. Thus, PLEKHA6 and PLEKHA7 take part in the junctional localization of PDZD11, while PLEKHA5 is responsible for its recruitment to microtubules.
Local, heterogeneous regions of active RhoA drive asymmetric junction contraction

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Convergent extension events during morphogenesis rely on the tight spatiotemporal control over cell-cell junction lengths. RhoA-mediated contractile actomyosin forces, acting at adherens junctions, alter junction lengths as a cyclic ratchet. During such ratcheted contractions, tricellular vertices exhibit sliding behavior, where a motile vertex contracts into a less motile vertex to facilitate asymmetric junction shortening. Failure to facilitate these dynamic, asymmetric junction behaviors results in convergent extension failures and embryonic lethality. While this phenomenon is well documented, the molecular and cellular origin of this asymmetry is unknown. To uncover the mechanism of asymmetric junction contraction, we use a bottom-up approach with the TULIP optogenetic system to exogenously target a RhoA activator, or RhoGEF, to drive local RhoA activation at cell-cell junctions in model Caco-2 epithelia. Upon uniform junctional RhoGEF localization, we find that this contraction culminates in a surprising and consistent contractile asymmetry. Using the predictive power of computational modeling, we examine the parameters that could specify these asymmetries. Our simulations predict that vertex asymmetry arises from the relative tensions at the tricellular vertices. However, when we experimentally measure vertex stiffness, we find no correlation, suggesting that asymmetry is independent of tissue mechanics. We find that this asymmetry is similarly independent of differential neighbor coupling and basal substrate tethering. By examining the local mechanochemical signaling acting at the junctions, we find that contractile asymmetries arise from stochastic regions of stable, persistent RhoA flares which are skewed to the less motile vertex. Consistent with this, activating half to a third of the junction drives asymmetric contraction where the localization of activation correlates with the less motile vertex. Additionally, at immobile vertices, E-cadherin is recruited to locally stiffen the vertex to slow its movement. Stable RhoA flares partition in regions of low interfacial E-cadherin and high plasma membrane, which also separate into anti-correlated microdomains along the junction. These membrane domains concentrate PIP2 and Anillin to create stochastic regions of stable RhoA that persist upon junction contraction. Here, Anillin increases RhoA’s residence time at the membrane, essentially protecting RhoA from GTP hydrolysis so that these sites can drive contractile asymmetry. Upon RhoA localization, RhoA recruits E-cadherin to immobilize and stiffen that vertex movement. Altogether these data decipher how these functional, contractile units are assembled and coordinated to respond to contractile forces during morphogenesis.

Junctional adhesion molecules, an unexpected journey into their structural properties

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One of the major functions of blood-tissue barriers is to seal vital organs from external cues and harmful substances in the environment. These barriers regulate homeostasis in tissues behind the tight junction (TJ)-barrier through restrictive paracellular diffusion. The blood-brain barrier (BBB) relies heavily on its TJ
to prevent dyshomeostasis. The TJ of the BBB is the strongest in the body. TJs are composed of mainly three membrane proteins: claudins, CLDN (a family of 27 members among mammalian species); occludin, OCLN; and junctional adhesion molecules, JAM (a family of four members). Research has shown claudins are primarily responsible for the barrier function of TJs but has failed to identify the precise role of JAMs nor their basic structural properties that make them essential for the formation of tight barriers. In our laboratory we have confirmed JAM’s secondary, tertiary and quaternary proteic structures for all members of the family. We have calculated the constants of affinity (K_D) for the formation of homotypic and heterotypic interactions. Additionally, we describe the effects of ionic strength and temperature in JAMs tertiary and quaternary structures. Finally, our strategy has identified the composition of the TJ in the BBB of mice, currently disputed in the literature. Taken together, our report enable research to quantitatively and qualitatively study TJ formation and function. Particularly in the BBB, our research demonstrates the need to further study the relationship between CLDN, OCLN and JAM proteins to protect the brain while enabling extravasation under physiological and pathophysiological conditions. Our results complete gapped threads in the unique role of JAMs in TJ formation and function.

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gH-625 liposome delivery PACAP through dynamic model of blood-brain barrier

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Pituitary adenylate cyclase-activating polypeptide (PACAP) has many effects in central nervous system (CNS) as neuroprotective tool, but as therapeutic agent, it has a rapid degradation, so it has been needed to develop nano-system to deliver functional PACAP in CNS; our system involves a peptide, gH-625, derived from glycoprotein H of the Herpes simplex virus 1: we functionalized liposomes with gH625 and then evaluated the ability of gH625-liposome loaded with rhodaminated PACAP to reach and cross an in vitro dynamic flow BBB model. This model is realized in a bioreactor, with a double flow chamber separated by a porous membrane; bEnd3 cells, were seeded on the porous membrane in the upper chamber. An anti-ZO-1 immunofluorescence confirmed that tight-junction of BBB were formed. For 2 hours we followed the delivery of gH625-liposomes loaded with PACAP: we showed an increase of gH625 liposome-PACAP in the lower chamber compared to non-functionalized liposomes-PACAP. Furthermore, we evaluated PACAP neuroprotection, treating dopaminergic neurons, seeded in the lower chamber with MPP+, a neurodegenerative agent. After 24h of exposure to MPP+, PACAP exerts neuroprotection at 10-8M. Finally, we demonstrated, by ELISA, an increase of PACAP concentration both in upper and lower chambers during time. Dopaminergic neurons were exposed to the full concentration (i.e. about 10-8M) of PACAP within 60 minutes. Our nanodelivery system results effective to expose dopaminergic neurons to suitable concentration of neuroprotective PACAP. Hence, this nanodelivery tool can be a suitable approach to protect neurons from injuries in neurodegenerative disease.
**Chaperones, Protein Folding, and Quality Control: Endoplasmic Reticulum & Golgi**

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**The Role of Calnexin in Regulating Proteostasis of RESET Substrates**
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RESET is a protein quality control pathway that involves the clearance of select misfolded secretory pathway proteins out of the endoplasmic reticulum (ER) to the Golgi via vesicular transport [1]. RESET substrates include misfolded, disease associated mutants of prion protein and other GPI-anchored proteins [1], and select transmembrane proteins including amyloid precursor protein. RESET contrasts with ER associated degradation and autophagy pathways, which retain misfolded proteins for degradation at the ER. During RESET, misfolded proteins are released by the ER-resident chaperone, calnexin (CNX), and requisitely associate with p24 or Tmp21 for vesicular transport to the Golgi. The misfolded proteins subsequently transit the cell surface en route to lysosomes where they are destroyed. Thus, steady-state turnover of RESET substrates is concomitant with ER-export. Furthermore, physiological and non-physiological chemical ER stressors dramatically enhance ER-export and consequent lysosomal degradation. Here we address the questions (i) what are the mechanisms regulating constitutive ER-export of RESET substrates during steady-state conditions and (ii) how do physiological ER-stress conditions enhance ER-export of RESET substrates for subsequent degradation? Our results suggest that the flux of RESET is regulated by competition of other unfolded/misfolded proteins for CNX-binding under steady-state conditions. Chemically blocking new expression of CNX substrates dramatically inhibits steady-state ER-export and degradation of RESET substrates. Conversely, an increase of competitor binding interactions with CNX during physiologically induced ER-stress conditions, including the upregulation of inflammatory glycoproteins, increases the flux of ER-export of RESET substrates. Critically, acutely triggering degradation of CNX by overexpressing E3 ligase Nixin/ZNRF4 causes the rapid release of RESET substrates out of the ER through the RESET pathway for downstream lysosomal degradation, which emphasizes CNX’s central role in regulating RESET. We present a new model that displacement from CNX by newly synthesized CNX substrates regulates the constitutive and stress-enhanced turnover of RESET substrates. These findings may offer insights into the connection between ER stress, inflammation, and the extracellular deposition of misfolded proteins as seen in prion and Alzheimer’s diseases. [1] Satpute-Krishnan et al. ER stress-induced clearance of misfolded GPI-anchored proteins via the secretory pathway. Cell. 2014 July 31

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**Er stress induced ersu cell cycle checkpoint impacts the nuclear functions via altering the dynamics of spindle pole body in saccharomyces cerevisiae**
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The endoplasmic reticulum (ER) is one of the biggest organelles spread extensively throughout the cytoplasm and a gateway to the secretory pathway involving in the production of secretory proteins and initiating lipid biosynthesis. The ER is continuous with the outer membrane of the nuclear envelope (NE) begs a fundamental question about how the functional capacity of the ER is coordinated with nuclear
architecture and functions. Previously, we reported that the ER Stress Surveillance (ERSU) cell cycle checkpoint ensures the only fully functional ER is inherited into daughter cells. The overwhelming of ER functional capacity such as an accumulation of unfolded proteins in the ER, is known to activate a cytoprotective signaling called the Unfolded Protein Response (UPR). Independently, ER stress also triggers a transit increase in phytosphingosine (PHS), which turns on the ERSU checkpoint, resulting in the temporally block of the cortical ER inheritance, Slt2 kinase phosphorylation, septin ring mislocalization from the bud neck leading to the cytokinesis delay. So far, we found that ER shaping protein Rtn1, Wsc1 and Slt2, mediating the ERSU checkpoint. In order to investigate if the ERSU events are coordinated with the nuclear function/membrane, we examined if ER stress impacts the spindle pole body (SPB) duplication and dynamics. Under the normal cell cycle, the sister chromatids movements and separation are controlled by the SPB. The SPB undergoes duplication followed by the insertion into the NE and establishment of the bipolar spindle, to attach and separate the sister chromatids in a timely manner. We found that ER stress did alter the dynamics of the SPB duplication/insertion into the NE, resulting in the changes in the spindle positioning and segregation. Specifically, upon duplication, the newly generated SPB was inserted into the NE with faster kinetics, occurring much earlier than G1/S phase. Furthermore, we found that the movement of duplicated SPB occurred with accelerated kinetics during S and G2/M phases. Importantly, we found that the ER stress-induced changes in the SPB require the ERSU component, SLT2, suggesting that SPB changes occurred as a part of the ERSU checkpoint. To further investigate molecular mechanisms of the ER stress-induced SPB changes, we investigated the involvement of the nuclear membrane components such as nucleoporins (Nups). I will discuss results of these and additional experiments.

Reticulons initiate the ER Stress Surveillance (ERSU) pathway activation

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The endoplasmic reticulum (ER) is essential for eukaryotic cell survival and has to be inherited from mother to daughter cells. It functions in the production and quality control of secretory proteins, lipid synthesis, and calcium storage. Therefore, it is critical that the ER is fully functional for optimal cell growth and survival. Previously, we identified the ER Stress Surveillance (ERSU) cell cycle checkpoint that ensures inheritance of functional ER. ERSU functions independently of the Unfolded Protein Response that induces transcription of ER chaperone coding genes and components that maintain ER homeostasis. ERSU activates the Slt2 MAPK to block cortical ER inheritance, translocate the septin ring away from the bud neck, and delay cytokinesis in response to ER stress (Babour et al. 2010). Recently, we discovered that the reticulon proteins, Rtn1 and Yop1, are important for the response and survival to ER stress (Piña et al. 2016). Cells lacking reticulon protein function failed to fully activate ERSU. Reticulon proteins are important factors that maintain the ER architecture by stabilizing membrane curvature. Moreover, this protein family has been implicated in cellular processes such as ER autophagy, highlighting their importance in cell growth and survival beyond their curvature stabilizing functions, although their mechanistic functions on the ERSU checkpoint have not been elucidated. We also found that ER stress elevates sphingolipid concentrations. The early sphingolipid biosynthetic product, phytosphingosine (PHS), activates ERSU when added exogenously (Piña et al. 2018). Other sphingolipids such as dihydrosphingosine failed to activate the ERSU events. PHS does not activate the UPR and fails to
activate the ERSU in reticulon knockout cells. These results suggest that PHS functions as an activating signal for the ERSU checkpoint in an Rtn1 and Yop1-dependent manner. Here, we investigate the molecular mechanism by which yeast reticulons mediate the response and survival to ER stress. We isolated Rtn1 and Yop1 mutants that do not activate ERSU, but retain the normal ER structural morphology. We also examined the role of reticulon proteins in the molecular mechanism of the ER inheritance block. We will discuss these results that point to Rtn1 and Yop1 as initial activating components of the ERSU pathway in response to the increased level of PHS.

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The GET pathway safeguards against non-imported mitochondrial protein stress
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The vast majority of the mitochondrial proteome, which contains over 1,000 proteins, is encoded in the nucleus and translated in the cytoplasm. Mitochondrial precursor proteins are imported into mitochondria by translocase complexes located in the outer and inner mitochondrial membranes through a process that depends on inner membrane potential. As a consequence of mitochondrial dysfunction, mitochondrial protein import is impaired and non-imported proteins accumulate outside of mitochondria. Previous studies found that non-imported mitochondrial proteins cause mitochondrial precursor overaccumulation stress (mPOS), which is highly toxic to the cell. In recent work, we and others have cataloged the localization and abundance of non-imported mitochondrial proteins in yeast experiencing mitochondrial impairment, and identified numerous distinct fates for non-imported precursors, including proteasomal destruction in the cytoplasm, nucleus, or at the mitochondrial surface, as well as delivery to the endoplasmic reticulum (ER) membrane. Although the ER is a destination to which a number of mitochondrial membrane proteins are delivered, it remains unclear how these proteins are targeted to the ER membrane. Here, we show that the guided entry of tail-anchored proteins (GET) complex, a known post-translational ER-insertion pathway for C-terminal tail-anchored proteins, plays an important role in protecting cells from mPOS by targeting mis-localized endogenous polytopic mitochondrial membrane proteins to the ER. Specifically, we find that loss of GET pathway components Get1, Get2 or Get3 prevents ER delivery of these mitochondrial proteins. Get3, the cytosolic ATPase of the GET pathway, physically interacts with non-imported mitochondrial membrane proteins. In the absence of a functional GET pathway, non-imported mitochondrial proteins destined for the ER are alternatively sequestered into Hsp42-dependent foci that associate with both mitochondria and the ER. Finally, we show that GET-dependent ER targeting of non-imported mitochondrial proteins prevents cellular toxicity and provides protein substrates for the ER-SURF pathway, which facilitates re-import of mitochondrial proteins from the ER. Overall, this study outlines an important role for the GET complex in maintaining cellular protein homeostasis in response to mitochondrial import failure.
Endoplasmic Reticulum Translocon Perturbation Modulates Toxicity Associated with Impaired Protein Quality Control

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The endoplasmic reticulum (ER) is the entry point for the majority of eukaryotic proteins functioning in the endomembrane system. Most proteins enter the ER via the transmembrane translocon complex. Translocon dysfunction can block access into the ER, which is detrimental to cellular health. Conserved translocon structure and function have been intensely studied in *Saccharomyces cerevisiae*. We found that an epitope tag on the central translocon pore, previously suggested not to impair translocon function, subtly impairs translocation in yeast cells. Strikingly, this tag also suppresses a phenotype associated with defective protein quality control, indicating a novel link between translocation and quality control. Ongoing work includes characterization of the effects of epitope tags on different translocon subunits, with the goal of identifying tags that minimally impede translocon function.

Cytokinesis: Mammalian

Generating a CRISPR tagging toolset to study cytokinesis in mammalian cells

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Cytokinesis separates a cell into two daughters at the end of mitosis due to the ingress of a contractile ring that pulls in the overlying plasma membrane. The use of gene editing tools has facilitated studies of cytokinesis in *C. elegans*, by generating endogenous tags and/or point mutations to study protein localization and function. However, due to the inefficiency of precise gene editing, these tools are not extensively used in mammalian cells, and cytokinesis studies continue to rely heavily on over-expressed transgenes and fixed cell imaging. In addition, most of our knowledge of mammalian cell cytokinesis comes from using HeLa cells, yet the mechanisms regulating cytokinesis vary widely between different cell types. Gene editing enables the study of cytokinesis proteins in their native context in more diverse cell types. Anillin is a scaffold that crosslinks actomyosin with the overlying membrane. It functions early in cytokinesis for ring positioning and later for ring-to-midbody transition, although its role in these processes has been challenging to study using exogenous tools. Ect2 is a RhoAGEF that is a master regulator of cytokinesis by activating RhoA to control assembly and ingestion of the contractile ring. Ect2 and RhoA have been notoriously difficult to visualize and study using transgenes. To overcome these limitations, we used CRISPR-Cas9 to successfully tag anillin and Ect2 with mNeonGreen in HEK293 cells, which localize similar to antibodies that report for endogenous proteins. We used the same constructs to also tag anillin with mNeonGreen in HeLa (human cervical cancer), HCT116 (human colorectal cancer), HepG2 (human hepatocellular carcinoma), and A549 (human lung carcinoma) cells. We then showed how this endogenous tag provides a useful tool to quantify the levels of anillin required to support different stages of cytokinesis by correlating levels of anillin with specific phenotypes after RNAi in HCT116 cells. We also are generating constructs to tag RhoA, as well as common markers including H2B histones, β-actin, non-muscle myosin IIb, β-tubulin and the plasma...
membrane with mRuby2 and/or TagBFP, to co-image with anillin and Ect2. Our constructs and protocols will be made available to the community as a toolset. These tools provide a way of directly visualizing key components of the cytokinesis machinery and will permit more functional studies in a broad range of cell types. Moreover, these tags can be used to study these proteins in other cellular processes that they have been implicated in, such as cell migration.

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Membrane compartmentalization of RhoGEF Ect2 and NuMA controls proper furrow initiation during cytokinesis
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Spindle elongation during anaphase is critical for proper chromosomes segregation. In human cells, plasma membrane localization of a large coiled-coil protein NuMA is crucial for spindle elongation by virtue of its ability to act as an adaptor for cortical dynein-dynactin. NuMA localization at the membrane in anaphase is dependent on its interaction with membrane lipids, in particular, PtIns(4)P and PtIns(4,5)P2. Notably, despite the presence of PtIns(4)P and PtIns(4,5)P2 across the entire cell membrane in anaphase, NuMA is excluded from the equatorial membrane. Earlier, we uncovered that Cyk4 (also known as MgcRacGAP) is necessary for excluding NuMA from the equatorial membrane. However, the mechanisms of NuMA exclusion by Cyk4 remain unclear. Here, we show that Cyk4 and its downstream component RhoGEF Ect2, but not Anillin, RhoA, and myosin, are required for NuMA exclusion from the equatorial membrane. Intriguingly, Ect2 interacts with the similar lipids to which NuMA binds. Therefore, we analyzed if the competition between Ect2 and NuMA for similar lipids is responsible for NuMA exclusion from the equatorial membrane. To test this, we generated stable cell lines expressing full-length Ect2 and a mutant Ect2 lacking lipid-binding potential. Notably, the expression of Ect2 mutant in cells lacking endogenous Ect2 fails in preventing NuMA accumulation at the equatorial membrane. This data supports the notion that NuMA and Ect2 compete for same lipid species. What is the biological relevance of NuMA exclusion from the equatorial membrane? NuMA-dependent dynnein-dynactin localization at the polar membrane ensures proper spindle elongation. Therefore, we analyzed the impact of equatorial membrane localization of NuMA and dynein-dynein on spindle elongation in Ect2 (RNAi). Surprisingly, Ect2 depletion only marginally affects chromosomes segregation and spindle elongation. Next, we sought to analyze the consequence on the equatorial Ect2 and cytokinesis by targeting NuMA at the equatorial membrane. Interestingly, artificially sending NuMA at the equatorial membrane negatively impact Ect2 and Rho levels, and significantly delays the furrow initiation. Thus, this work establishes a new paradigm whereby membrane compartmentalization of RhoGEF Ect2 and an essential mitotic regulator NuMA is critical for temporal regulation of furrow initiation during cytokinesis.

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Knockout of Cep55 suggests existence of alternative mechanisms for abscission, and cell-type specific responses to cytokinesis failure
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Cytokinetic abscission severs the daughter cells and is mediated by the midbody. The prevailing model, based on work in cell lines, places the coiled-coil midbody protein Cep55 at the top of the abscission cascade in vertebrate cells, recruiting endosomal sorting complexes required for transport (ESCRT) components to accomplish membrane scission. Here, we sought to elucidate the roles of Cep55 in abscission and development, by analyzing the cellular and developmental consequences of Cep55 loss in vivo. We show knockout of Cep55 in mice causes reduced embryo size and postnatal lethality. The brain is disproportionately affected, with a severe deficit in neurogenesis. Cerebral cortex NSCs undergo a polarized form of cytokinesis that is developmentally regulated, with severing of the midbody at the apical membrane. Quantitative and live imaging analyses of abscission in Cep55−/− cortical NSCs show that it is prolonged but still occurs in most cells, and ESCRT recruitment is decreased but not eliminated. A subset of NSCs fail cytokinesis and become binucleate. Abscission defects are not specific to NSCs, but also occur in Cep55−/− cultured primary embryonic fibroblasts. Thus, Cep55 is not absolutely required for ESCRT recruitment or abscission in vertebrate cells, but plays an important regulatory role that increases the speed and success rate. In addition, we show abscission failure causes tissue-specific responses that contribute to the microcephaly phenotype: binucleate NSCs elevate p53 but binucleate fibroblasts do not. This leads to massive apoptosis in the brain, but not other tissues. This work highlights the need for additional in vivo studies of the abscission machinery to clarify in vitro models, and adds to emerging evidence that abscission regulation and error tolerance vary by cell type and are especially crucial in neural stem cells as they build the brain.

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Identification of lipids bound to membrane-associated cytokinesis proteins
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As a cell divides, it must rearrange and separate its membrane-bound organelles as well as its plasma membrane to create equivalent daughter cells. Cells actively maintain complex and diverse lipidomes that encompass many thousands of lipids, which reside in these membrane-bound structures. We have shown that the lipid composition of dividing cells is altered relative to non-dividing cells. However, the roles of these lipids remain largely unexplored. It is our hypothesis that specific interactions between lipids and proteins might contribute to their functions during cytokinesis. The first step to test this hypothesis is to determine if specific lipids are bound to cytokinetic proteins. We developed a technique to systematically explore protein-lipid interactions in cytokinesis. This involves detergent free lysis to obtain small membrane fragments from cells expressing the protein of interest bound to GFP, followed by GFP trap pulldowns and identification of bound lipids by liquid chromatography-mass spectrometry (LC-MS). We validated this approach in HeLa cell lines stably expressing Sec61B, a known ER transmembrane protein, Lact-C2-GFP, a phosphatidylserine (PS) binding protein, or TOMM20-GFP, a protein expressed on the outer mitochondrial membrane. All samples resulted in pulldown of expected lipids, validating our experimental setup. Many cytokinetic proteins are associated with the plasma membrane, including RACGAP1 and CHMP4B. We have identified which lipids specifically associate with these proteins and will next conduct functional studies.
The nature of mitotic forces in epithelial monolayers
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Epithelial cells undergo vast morphological changes during division as, a spread parent cell transitions to a rounded morphology, the mitotic cell elongates and constricts at the center from metaphase to cytokinesis, and finally as the daughter cells reintebrate into the epithelium. These changes occur despite dividing cells being confined by neighboring cells, indicating the need for extracellular force generation. While the forces generated during mitotic rounding are now well understood, the nature of the forces generated after mitotic rounding remain unknown. Here we studied MDCK and MCF10A epithelial monolayers, and measured changes in cell-cell stress during division. We identified two distinct stages of force generation that follow mitotic rounding: (1) protrusive forces along the mitotic axis that drive mitotic elongation, and (2) more uniform outward forces associated with post-mitotic re-spooling. Next, we sought to determine the origin of forces generated during mitotic elongation. Neighboring cells could in principle generate these forces. However, neighboring cells were randomly positioned with respect to the dividing cell’s axis, did not display any consistent movements or adhesion remodeling, and perturbation of cadherin-based adhesions did not diminish observed mitotic forces. Elongation of the dividing cell, however, was accompanied by deformation of adjacent cells exclusively along the mitotic axis, suggesting that mitotic elongation is driven by forces originating from the dividing cell. Thus, we examined the mechanisms of protrusive force generation within the dividing cell. Volume during division was nearly conserved, and computational modeling showed that with near volume conservation, contraction of the cytokinetic ring should enhance cell elongation. Consistent with this, pharmacological inhibition of cytokinetic ring contraction at metaphase reduced cell elongation and chromosome separation, indicating that extracellular forces powering mitotic elongation originate primarily from cytokinesis, and are important not only for elongation, but also to properly separate genetic material. Deformation of adjacent cells during mitotic elongation was observed in epithelia from Drosophila, C. elegans, mouse, and Xenopus model organisms as well. Our results show that cells dividing within epithelial monolayers generate distinct forces in three mitotic stages: rounding, elongation, and spreading, and that force generation during mitotic elongation is a fundamental aspect of cell division within epithelia.
Protocadherin 7 localizes to the plasma membrane during mitosis and promotes cytokinesis by a palmitoylation-dependent mechanism

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Successful cell division requires dramatic reorganization of the cell cortex in coordination with actomyosin cytoskeleton organization, membrane trafficking and cell adhesion. Although the contractile actomyosin ring is considered as hallmark of cytokinesis, in some cell types cell adhesion systems have been shown to drive cytokinesis independently from actomyosin function. We previously reported that Protocadherin 7 (PCDH7) localizes to the mitotic cortex which is required for building up the full mitotic rounding pressure. Here, we show that PCDH7 localizes to the mitotic cell cortex and to the cleavage furrow by a palmitoylation-dependent mechanism. At the cleavage furrow, PCDH7 facilitates the activation of myosin II and successful cytokinesis. Strikingly, PCDH7 promotes cytokinesis even when the myosin II contractility and integrin mediated adhesion are blocked. This work describes a palmitoylation-dependent cortical reorganization which promotes cytokinesis under different conditions.

Clic4 and clic1 bridge plasma membrane and cortical actin network for a successful cytokinesis


CLIC4 and CLIC1 are members of the well-conserved chloride intracellular channel proteins (CLICs) structurally related to omega-type glutathione-S-transferases. In this study, we aimed to elucidate the new roles of CLICs in cytokinesis (Uretmen Kagiali et al., 2020). At the onset of cytokinesis, CLIC4 accumulates at the cleavage furrow and later localizes to the midbody in a RhoA-dependent manner. The cell cycle-dependent dynamic localization of CLIC4 is abolished when its glutathione S-transferase activity-related residues (C35A and F37D) are mutated. A systematic comparison between proximity interactomes of wild-type and mutant CLIC4 identified novel interactors of CLIC4 in cytokinesis. Further analysis revealed the association of ezrin, anillin, and ALIX with CLIC4 during cytokinesis. Strikingly, a positive feedback loop is found to regulate ezrin phosphorylation and cleavage furrow localization of CLIC4. Whereas CLIC4 is involved in the ezrin activation at the cleavage furrow, the inhibition of ezrin phosphorylation diminishes the translocation of CLIC4 to the cleavage furrow. Furthermore, the knockouts of CLIC4 and CLIC1 cause abnormal blebbing at the polar cortex and regression of the cleavage furrow during cytokinesis leading to multinucleated cells. Both CLIC4 and CLIC1 accumulate at retracting membrane blebs during cytokinesis suggesting a role in the regulation of polar blebs. We conclude that CLICs function together with ezrin where they bridge plasma membrane and actin cytoskeleton at the polar cortex and cleavage furrow to ensure cortical stability and successful completion of cytokinesis in mammalian cells. Reference: Uretmen Kagiali, Z.C. et al. 2020. "CLIC4 and CLIC1 bridge plasma membrane and cortical actin network for a successful cytokinesis", Life Science Alliance, 3 (2), e201900558. http://dx.doi.org/10.26508/lsa.201900558
Investigating WNT signaling dynamics in response to AXIN2 knockdown in HT29 Colorectal Cancer Cells
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Increased WNT/beta-catenin signaling pathway has been well-associated with colorectal cancer (CRC). The negative feedback loop that keeps beta-catenin levels in check in normal cells was observed to be dysregulated in CRC cells. This is fundamentally due to a mutation in adenomatous polyposis coli (APC), which is responsible for degradation of beta-catenin. The role of APC is well-known in this regard however, AXIN2, another component of this negative feedback loop is less known and yet has been recently recommended as a clinical marker for poor CRC prognosis. Therefore, we seek to explore how AXIN2 functions as part of the negative WNT/beta-catenin signaling feedback loop and provide understanding of its implication in CRC prognosis. Our bioinformatics analysis suggests that CRC cells carrying APC-truncating mutations have only a minor decrease in enzymatic efficiency of the destruction complex, but a much more significant reversal in the correlation between AXIN2 and APC expression level. Therefore, we hypothesize that the interaction between truncated-APC and AXIN2 is responsible for the dysregulation of the feedback loop, we transfected HT29 cells with TCF/LEF reporter and AXIN2 siRNA, the result indicated that the WNT/beta-catenin signaling activity is increased upon AXIN2 knockdown, which confirmed the functional integrity of part of the WNT pathway from destruction complex to transcription activation by TCF/beta-catenin complex, narrowing down the malfunctioning component(s) to within the destruction complex. Protein analysis further demonstrated that upon AXIN2 siRNA-mediated knockdown, a selection of WNT/beta-catenin targeted genes, including c-JUN and c-MYC, fluctuate according to their interactive relationship with AXIN2 in canonical WNT pathway. This indicates that AXIN2 fluctuations have potential effects in stress-induced apoptosis and cellular proliferation, which explains the efficacy of using AXIN2 as a prognostic marker. Finally, using cell confluency data collected during a time-course of siRNA treatment, we were able to measure and compare changes in cell number between AXIN2 knockdown and control groups. When AXIN2 expression is low, the cell proliferation rate is reduced. Overall, our results indicate that AXIN2 serves a pivotal role in cancer development and its interaction with truncated APC is most likely responsible for the dysregulation of the negative feedback loop that fine tunes the beta-catenin level.

ER and Golgi Transport

Bottom-up approach reveals retrotranslocase activity of the membrane-embedded ubiquitin ligase Doa10 in ER-associated protein degradation
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In the endoplasmic reticulum (ER), proteins of the secretory pathway fold and assemble into multiprotein complexes. A quality control pathway termed ER-associated protein degradation (ERAD) removes unassembled subunits and terminally misfolded proteins. Substrates are ubiquitinated by conserved membrane-embedded ubiquitin ligase complexes, extracted from the membrane by the AAA ATPase Cdc48, and degraded by the proteasome. How substrates containing hydrophobic
transmembrane segments and luminal potentially folded domains are removed from the membrane is unclear. To gain mechanistic insight into this process we set up a reconstituted system with purified proteins and artificial phospholipid vesicles (liposomes). Reconstitution of multiple membrane proteins into liposomes is often hampered by low co-reconstitution efficiencies and incompatibility of different detergents used for different membrane proteins. To overcome these challenges, we reconstituted components of the ERAD machinery first into separate liposomes together with SNARE fusogens. SNARE-mediated membrane fusion then resulted in efficient co-reconstitution. Importantly, this system ensures that interaction of membrane proteins only occurs in a lipid bilayer, thus avoiding potentially non-native interactions in detergent-stabilized solutions. Using this system we show that the ubiquitin ligase Doa10 is a retrotranslocase that facilitates the movement of a membrane protein into the cytosol. Doa10 only accommodates substrates in an unfolded state. Cdc48 action unfolds luminal segments of the substrates thus enabling their retrotranslocation. Our results reveal that membrane protein extraction relies on the interplay of a cytosolic AAA ATPase and a membrane-embedded retrotranslocase. Moreover, we provide a useful tool for the mechanistic investigation of membrane-bound processes.

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Snrbp, A spliceosomal checkpoint in proteostasis
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There is mounting evidence that secretion and autophagy are intimately linked cellular processes. The secretory pathway serves as a platform as well as a source of membranes and proteins to growing phagophore. However, how autophagy influences membrane trafficking is poorly understood. Here, we screened a library of 124 putative autophagy regulators using for their effects on the ER-to-Golgi trafficking. As a read-out, we monitored ER-to-Golgi trafficking of Mannosidase-II using the RUSH system. SNRPB, a spliceosomal component, is one of the top hit of our RNAi screen. We found that SNRPB arrests ER-to-Golgi trafficking and decrease ERES, upon knockdown. In our RNAseq analysis, SNRPB depletion impairs the splicing of COPII components. In response to increased ER load by the over expression of GAT1, the level of SNRPB is elevated, in order to facilitate the efficient splicing of COPII machinery. We also observed that SNRPB knockdown abolishes, increment in the number of ERES, in response to ER load. The elevated level of SNRPB under high ER load is dependent on ATF6.

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O-glcnacylation plays a role in sec24 function
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Coat protein complex II (COPII) mediates forward protein and lipid trafficking from the endoplasmic reticulum via five proteins required for in vitro vesicle formation: SAR1, SEC13, SEC23, SEC24, and SEC31. While the structure and function of COPII proteins are well understood, we do not fully understand how COPII is regulated in response to environmental and cellular stimuli. We and others have found that O-
linked β-N-acetylglucosamine (O-GlcNAc), a dynamic single-sugar modification added to serines and threonines of intracellular proteins, decorates many human COPII components, including all four human SEC24 paralogs. Additionally, others have shown that COPII, and specifically SEC24, may play a role in the formation of autophagosomes. However, the regulation of SEC24’s canonical and autophagic functions remains unclear. To determine the function of SEC24 O-GlcNAcylation, we mapped O-GlcNAc sites on SEC24C and SEC24D using mass spectrometry and created unglycosylatable serine or threonine to alanine mutations at each O-GlcNAc site. We then used CRISPR-Cas9 to delete SEC24C and SEC24D cell lines from various cell types. We show that both SEC24C and SEC24D O-GlcNAcylation increases within minutes of treatment with an O-GlcNAcase inhibitor, suggesting that O-GlcNAcylation may play a regulatory role on SEC24. Ongoing work aims to determine the role of this dynamic modification on trafficking of multiple cargoes. Our results enhance our understanding of the role of O-GlcNAc in COPII trafficking and SEC24 function.

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Calcium Sensors ALG-2 and Peflin Bind ER Exit Sites in Alternate States to Modulate Secretion at Steady State and in Response to Calcium Signaling

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While the essential machinery for ER-to-Golgi transport is well-characterized, important questions remain about how trafficking is regulated. Our lab has shown that ER luminal calcium strongly modulates ER export. We have focused on the penta-EF-hand (PEF) Ca2+ sensors apoptosis-linked gene 2 (ALG-2) and peflin as potential effectors that translate Ca2+ changes into trafficking changes via interactions with the COPII coat. We employed a series of knockdown and overexpression experiments coupled with quantitative trafficking and targeting assays to characterize the PEF proteins at ERES. We found that both ALG-2 and peflin bind to ER exit sites (ERES), but that peflin both required ALG-2 for binding and antagonized ALG-2 binding there. Furthermore, we found that adjustment of the ALG-2:peflin cellular expression ratio produced commensurate ratio changes at ERES and increased or decreased transport by over 100% of the basal ER export rate. Less peflin and increased ALG-2 at ERES increased transport above basal, while more peflin and less ALG-2 at ERES inhibited transport below basal, implying that the transport rate under basal conditions is a balance of both stimulatory and inhibitory PEF protein effects. The functional effects were extended to multiple actively sorted COPII cargo types, including transmembrane cargo, GPI-anchored proteins, and procollagen I. However, opposite effects occurred for unsorted bulk flow cargo, demonstrating that PEF proteins regulate the COPII sorting function as opposed to simply vesicle production. We also discovered a novel PEF protein-mediated secretory response to demanding physiological conditions. Sustained agonist-driven ER Ca2+ signaling depressed ER export rates by 40 percent below basal - a process that strictly required ALG-2. During this response, targeting of the COPII outer coat to ERES is decreased and targeting of peflin is increased, though in this case peflin is not required for the transport inhibition. Rather, under these...
conditions, ALG-2 may recruit another, unknown binding partner that alters COPII targeting and function in a distinct manner. In summary, PEF protein binding to ERES regulates ER export at steady-state and during sustained Ca2+ signaling to produce overlapping but distinct functional effects.

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GRASP55 regulates huntingtin unconventional secretion and toxicity
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Two Golgi peripheral membrane proteins GRASP55 and GRASP65 were originally identified to play essential roles in the regulation of Golgi stack formation and ribbon linking. Recent reports showed that GRASPs, especially GRASP55, regulate unconventional secretion of cytoplasmic proteins that lack obvious signal sequences. It is not clear how a Golgi protein such as GRASP55 controls unconventional protein secretion (UPS), which is Golgi independent. Here, we tested the secretion of cytosolic neurodegenerative toxic proteins including huntingtin (Htt), α-synuclein, tau, and TDP43. Our results showed that at least Htt and SOD1 are secreted. Further analysis using Htt exon 1 with a long polyglutamate (polyQ) stretch (Htt-Q74) as a model system showed that Htt UPS depends on GRASP55 but not GRASP65. Interestingly, Htt-Q74 UPS is enhanced under stress conditions such as energy, nutrient starvation and ER stress. Htt-Q74 secretion is autophagy dependent and blockade of its secretion results in accumulation of Htt aggregates in the cell. GRASP55 regulates the secretion of Htt by functioning as a tether and GRASP55 autophagy defective mutant F37A can’t rescue the secretion of Htt. Moreover, GRASP55 is upregulated under amino acid starvation, ER stress and also in Htt knock-in mouse. Our model is GRASP55 functions as a stress sensor to regulate unconventional secretion and protein homeostasis.

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Investigating Molecular Mechanisms Regulating Localization of Arf Family Proteins and Secretion of Insulin Like Growth Factor
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The insulin-like growth factor 2 (IGF2) plays critical roles in cell proliferation, growth, migration, differentiation and survival that are important for various physiological processes. Defects in IGF2 expression are related with various growth disorders and tumorigenesis. One of the physiological process that is regulated by IGF2 is skeletal myogenesis. The expression of IGF2 is under tight controlled at the transcriptional level, at mRNA level and at the translational level. Newly synthesized IGF2 needs to be secreted out of the cell to perform its physiological functions. Despite its importance, the molecular mechanisms mediating secretion of IGF2 remain unclear. Here we utilized a RUSH assay to analyse secretion of IGF2. Our suggests that the second half IGF2 (aa: 98-180) contain motifs that are sufficient for exporting IGF2 out of the ER. Interestingly, the secreted form of IGF2 was sensitive to glycosidase treatment suggesting that only the glycosylated form of IGF2 can be secreted. We found that a type I transmembrane protein, TMED10, is essential for secretion of IGF2 and for C2C12 cell differentiation. TMED10 is a member of the p24 family. The p24 family proteins cycle between the ER and the Golgi and some of p24 family members are shown to function as cargo receptors to regulate ER export of specific GPI-anchored proteins in mammalian cells. KD of TMED10 dramatically decreased the
secretion level of IGF2 in the RUSH assay. Our GST pull down result indicates that TMED10 binds IGF2. We then reconstituted release of IGF2 into COPII vesicles and this assay suggests that TMED10 regulates package of IGF2 in COPII vesicles to be efficiently delivered to the Golgi. Moreover, our analysis indicates that TMED10 is important for packaging a variety of Golgi-localized glucosidases catalyzing the O-glycosylation process into transport vesicles. Based on these results, we hypothesize that TMED10 functions as a cargo receptor to mediate packaging of IGF2 into COPII vesicles to be efficiently delivered to the Golgi and TMED10 is also important for delivery of Golgi enzymes to the Golgi for O-glycosylation modifications on IGF2 in order for IGF2 to be secreted.

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The endoplasmic reticulum forms a reticular structure around centrosomes in *C. elegans* embryos
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Centrosomes serve as microtubule organizing centers (MTOC) that nucleate microtubules. Throughout most of the cell cycle of the *C. elegans* embryo, centrosomes are surrounded by a membranous structure that is visible by microscopy using a fluorescently tagged endoplasmic reticulum (ER)-associated protein. Similar structures were also observed in *Drosophila*, Sea Urchins, *Xenopus* egg extracts and human tissue culture cells. The ER is a highly dynamic organelle composed of membrane tubules and sheets found throughout the cell. However, how it reorganizes around centrosomes, its conformation at this site and the role of this membranous structure are not known. Our goal is to understand the mechanism of this membrane accumulation and determine its function. To determine the organization of this pericentrosomal membranous structure, we used Focused Ion Beam-Scanning Electron Microscopy (FIB-SEM) and examined these membranes in *C. elegans* embryos at a nanometer-scale resolution. Our data show that the membrane around the centrosome forms an inter-connected reticulum of membrane tubules, leading us to name this structure the pericentrosomal reticulum (PCR). To better describe this structure's dynamic nature, 1-cell embryos were imaged over the course of the cell cycle. We found that PCRs are first detected immediately following centrosome duplication, and they increase in size from interphase to metaphase, corresponding to the increase in the size of the pericentrosomal material (PCM) they encompass. To buildup ER around the MTOC, the cell likely uses a motor protein in the membrane rearrangement process. To test this, we performed RNAi against the motor protein dynein and found that it does not alter the reticulum's accumulation. However, our RNAi screen results suggest that actin may contribute to this reorganization. The PCR is present in every embryonic cell division cycle in *C. elegans*, suggesting that it has a role in its proper execution. We speculate that it contributes to an environment for adequate centrosome function and/or connects the centrosomes to the nuclear envelope.
**Role of COPI Coat in Procollagen Export from Endoplasmic Reticulum**
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The mechanism of large secretory cargo export from Endoplasmic Reticulum has been debated for the last several decades, procollagen precursor of collagen being often used as the prototypical cargo in these studies. The traditional secretion model postulates procollagen export from ER exit sites (ERESs) in COPII-coated vesicles and repackaging into COPI vesicles at an intermediate ER-Golgi compartment (ERGIC) for subsequent delivery to cis-Golgi. We recently visualized bona fide transport intermediates delivering type I procollagen from ERES to cis-Golgi by fast (0.5-1 s/frame), super-resolution (120 nm) imaging of live cells. Surprisingly, these intermediates had no COPII coat and did not appear to stop at any distinct intermediate compartment on their way to cis-Golgi. Furthermore, their export from ERES appeared to be blocked by brefeldin A, an inhibitor of GDP->GTP exchange at ARF1 GTPase that is required for ARF1 recruitment to membranes and COPI coat formation. At the same time, brefeldin A did not inhibit the delivery of procollagen from the ER to lysosomes. To understand these observations, we further examined ARF1 and COPI coatomer localization at procollagen ERES and ER-Golgi transport intermediates. Colocalization experiments in 3D and fast, super-resolution time-lapse imaging in 2D suggested procollagen export from ERES in COPI rather than COPII coated transport intermediates. Taken together with other reported observations, our findings suggest that procollagen transport intermediates form at distal ERES regions upon ARF1 activation and COPI coat recruitment, although further studies are needed to fully characterize the COPI coat function in this process.

**Kinases and Phosphatases**

**Redox priming promotes Aurora A activation during mitosis**
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Cell cycle-dependent redox changes can mediate transient covalent modifications of cysteine thiols to modulate the activities of regulatory kinases and phosphatases. Our previously reported finding that
protein cysteine oxidation is increased during mitosis relative to other cell cycle phases suggests that redox modifications could play prominent roles in regulating mitotic processes. The Aurora kinases and their downstream targets are key components of the cellular machinery that ensures the proper execution of mitosis and the accurate segregation of chromosomes to daughter cells. In this study, X-ray crystal structures of the Aurora A kinase domain delineate redox-sensitive cysteine residues that, upon covalent modification, can allosterically regulate kinase activity and oligomerization state. We show in both *Xenopus laevis* egg extracts and in mammalian cells that a conserved cysteine residue within the Aurora A activation loop is crucial for Aurora A activation by autophosphorylation. We further show that covalent disulfide adducts of this activation loop cysteine promote autophosphorylation of the Aurora A kinase domain. These findings reveal a potential mechanistic link between Aurora A activation and changes in the intracellular redox state during mitosis, as well as insights into how novel small molecule inhibitors may be developed to target specific subpopulations of Aurora A.

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**Single-cell AMPK responses to OXPHOS inhibition reveal heterogeneity in glycolysis and ATP turnover**

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Oxidative phosphorylation (OXPHOS) generates the majority of cellular ATP. Inhibitors of OXPHOS induce metabolic stress and have clinical uses in diabetes and cancer. To identify determinants of OXPHOS sensitivity in single cells, we used live-cell reporters for ATP, ADP/ATP, or activity of the energy-sensing kinase AMPK. We found that AMPK and ADP/ATP respond heterogeneously regardless of the inhibitor or dose used, revealing underlying variation in cellular metabolism. While ATP concentration was maintained homeostatically, ADP/ATP and AMPK responses were determined by the rates of glycolysis and ATP turnover. Increasing insulin/AKT signaling activity or reducing protein translation promote a cell state in which AMPK is resistant to activation by OXPHOS inhibition. Variable AMPK responses are propagated to the growth signaling pathways mTORC1 and ERK, known targets of AMPK inhibition. Our analysis provides a basis for predicting the resistance of multicellular populations to OXPHOS inhibition.
Regulation of fibroblast polarity by Src tyrosine kinase

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Src is a tyrosine-phosphorylated kinase located under the plasma membrane, which is activated during cell adhesion, migration, and elongation. The Src family may play important roles in determining the polarity of cells during cell extension and elongation because of its strong involvement in signal transduction between inside of the cell and extracellular matrix proteins. However, the mechanism underlying Src-mediated polarity formation remains largely unknown. In the present study, we investigated the mechanism of Src tyrosine kinase-induced cell polarity formation and cell elongation using Src family (Src, Yes, Fyn)-knockout fibroblasts (SYF cells; ATCC, Manassas, VA) together with a Src family tyrosine kinase inhibitor (Src Inhibitor No. 5; Biaffin, Kassel, Germany). SYF cells cultured on a glass surface were observed to elongate symmetrically into spindle-shaped cells, and form small focal adhesions at both ends of the cells. In addition, SYF cells transfected with the wild-type c-Src gene showed almost the same morphology as normal fibroblasts, with the formation of pseudopods at the leading edge. When normal fibroblasts were treated with a c-Src inhibitor, they were observed to elongate into symmetrical, elongated spindle-shaped cells, similar to SYF cells. After washout of the c-Src inhibitor, the cells actively elongated again, with recovery of their pseudopods and showed almost the same morphology as normal fibroblasts. These results suggest that the polarity of cells during extension and elongation may be regulated by the Src family, and that the expression and regulation of Src are important for the formation of polarity during cell elongation.

EMAP II modulated SPHK1 expression and translocation

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Bioactive sphingolipid sphingosine-1-phosphate (S1P) is a signaling mediator involved in inflammation, proliferation, and angiogenesis. The molecular mechanisms regulating S1P burden are poorly understood. Similarities between two gene transcript activation profiles of ‘find me’ pro-inflammatory mediators, S1P and Endothelial Monocyte Activating Polypeptide (EMAP) II suggested a strategic link between their signaling pathways. In this study, we determined that EMAP II triggers a bimodal phosphorylation, transcriptional regulation and membrane translocation of S1P synthesis catalyzing enzyme, Sphingosine Kinase 1 (SPHK1) through a common upstream process in both macrophages and smooth muscle cells (SMCs). EMAP II initiates a dual function of ERK1/2: phosphorylation of SPHK1 and regulation of the transcription factor EGR1 that induces expression of SPHK1. Activated ERK1/2 induces a bimodal phosphorylation of SPHK1 which reciprocally increases S1P levels. This identified common upstream signaling mechanism between a protein and a bioactive lipid initiates cell specific downstream signaling representing a multifactorial mechanism that contributes to inflammation and SMC proliferation.
A screen for conserved kinase regulators of tubulogenesis in *C. elegans* reveals a role for the Par1-like gene *pig-1* and putative endothelial functions for its human ortholog *MELK*.

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Tubulogenesis, the process by which organisms form biological tubes, plays an integral role in blood vessel formation and function. Defects in tubulogenesis can lead to congenital disease (e.g., capillary malformations) and dysregulated tubulogenesis associated with tumor-induced angiogenesis promotes cancer progression and metastasis. Kinases are critical regulators of key cellular functions and signal transduction pathways, with roughly 30% of human proteins being phosphorylated by kinases. They are attractive targets for therapeutic development; therefore, finding kinases involved in vascular tubulogenesis is an important translational research goal. The *C. elegans* excretory canal (*ExCa*), a large single-celled tube, provides a tractable model to study tubulogenesis. Human orthologs of several genes that regulate *ExCa* tubulogenesis have been implicated in vascular disease and/or regulation of angiogenic behaviors. Therefore, we hypothesized that we could discover new conserved kinase regulators of angiogenesis by screening for new players in *ExCa* tubulogenesis. Using OrthoList, a compendium of *C. elegans* genes with human orthologs (ortholist.shaye-lab.org), we identified 248 *C. elegans* kinases with human orthologs. An initial RNAi screen against these led to discovery of eight whose loss causes *ExCa* defects. Of these eight, four were known to have roles in tubulogenesis or angiogenesis (indicating a low false-negative rate in the screen). To define the function of the four new kinase regulators of *ExCa* tubulogenesis we are characterizing their phenotypes in *C. elegans* using fluorescent reporters that mark the cytoskeleton and cellular organelles. Published expression profiles of murine vasculature suggest that orthologs of all the kinases identified in our screen are expressed in endothelial cells. However, four of these (*hpo-11, kin-18, mrck-1* and *pig-1* in *C. elegans*) have never been studied for their role in endothelial biology. We found that orthologs of these four are expressed in human umbilical vein endothelial cells (HUVEC); a canonical model for studying endothelial cell behavior and angiogenesis *in vitro*. We are first focusing on the kinase MELK, the ortholog of *C. elegans pig-1*, because this kinase has been intensively targeted for therapeutic intervention even though very little is known about its physiological function. We are using shRNA to knockdown MELK in HUVEC and performing various endothelial and angiogenesis assays to define MELK function. We expect that combining primary human endothelial cell culture studies with *in vivo* *C. elegans* genetic and cell biological approaches will allow us to define a conserved role for MELK (and other kinases) in angiogenic tubulogenesis.

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**Ga/GSA-1 works upstream of PKA/KIN-1 to regulate calcium signaling and contractility in the *Caenorhabditis elegans* spermatheca**

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Correct regulation of cell contractility is critical for the function of many biological systems. The reproductive system of the hermaphroditic nematode *C. elegans* contains a contractile tube of myoepithelial cells known as the spermatheca, which stores sperm and is the site of oocyte fertilization. Regulated contraction of the spermatheca pushes the embryo into the uterus. Cell contractility in the
spermatheca is dependent on actin and myosin and is regulated, in part, by Ca\textsuperscript{2+} signaling through the phospholipase PLC-1, which mediates Ca\textsuperscript{2+} release from the endoplasmic reticulum. Here, we describe a novel role for GSA-1/Gα\textsubscript{s}, and protein kinase A, composed of the catalytic subunit KIN-1/PKA-C and the regulatory subunit KIN-2/PKA-R, in the regulation of Ca\textsuperscript{2+} release and contractility in the C. elegans spermatheca. Without GSA-1/Gα\textsubscript{s} or KIN-1/PKA-C, Ca\textsuperscript{2+} is not released, and oocytes become trapped in the spermatheca. Conversely, when PKA is activated through either a gain of function allele in GSA-1 (GSA-1(GF)) or by depletion of KIN-2/PKA-R, the transit times and total numbers, although not frequencies, of Ca\textsuperscript{2+} pulses are increased, and Ca\textsuperscript{2+} propagates across the spermatheca even in the absence of oocyte entry. In the spermathecal-uterine valve, loss of GSA-1/Gα\textsubscript{s} or KIN-1/PKA-C results in sustained, high levels of Ca\textsuperscript{2+} and a loss of coordination between the spermathecal bag and sp-ut valve. Additionally, we show that depleting phosphodiesterase PDE-6 levels alters contractility and Ca\textsuperscript{2+} dynamics in the spermatheca, and that the GPB-1 and GPB-2 Gβ subunits play a central role in regulating spermathecal contractility and Ca\textsuperscript{2+} signaling. This work identifies a signaling network in which Ca\textsuperscript{2+} and cAMP pathways work together to coordinate spermathecal contractions for successful ovulations.

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Dgkθ Is involved in cell proliferation of mouse embryonic fibroblasts and affects fetal development. K. Ito\textsuperscript{1}, S. Ueda\textsuperscript{1}, M. Yamaguchi\textsuperscript{1}, M. Ikawa\textsuperscript{2}, M. Yamanoue\textsuperscript{1}, Y. Shirai\textsuperscript{1}; \textsuperscript{1}Department of Agrobioscience Kobe University, Kobe, JAPAN, \textsuperscript{2}Research Institute for Microbial Diseases, Osaka, JAPAN.

Diacylglycerol kinase (DGK) regulates protein kinase C signaling by converting diacylglycerol into phosphatidic acid. Of the 10 DGK isoforms, DGKθ regulates postsynaptic functions in neurons, but its role in other tissues and organs are still unclear. The DGKθ gene is located at 4p16.3 at the end of the short arm of chromosome 4, and its deletion is known to cause Wolf-Hirschhorn syndrome (WHS), which is a genetic disorder that is characterized by delayed growth and development. In this study, to investigate the role of DGKθ in the developmental stage, we employed mRNA silencing approach and DGKθ knockout (KO) mice. Knockdown of DGKθ mRNA by siRNA reduced the rate of cell proliferation in mouse embryonic fibroblasts (MEFs). In knockdown cells, the expression of EGF receptor was lower than wild-type cells. Next, we generated DGKθ KO mice and verified their embryonic phenotype. DGKθ KO mice showed weight loss compared to the littermates of wild-type fetuses (E14.5 and E18.5). This weight loss was observed continuously until ten weeks of age. While there was no embryonic lethality observed in DGKθ KO mice, they exhibited fetal growth retardation (FGR). Whole-mount embryo staining showed no abnormalities in embryonic organ development. MEFs derived from KO mice revealed a decreased rate of cell proliferation and reduced expression of the EGF receptor and its downstream signaling as compared to wild-type mice. These results suggest that DGKθ is involved in MEF cell proliferation and contributes to fetal development.
Kinesin/Dynein Interplay

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Huntingtin Phosphorylation Increases Processivity of Rab5 Cargoes
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Huntingtin phosphorylation at site serine 421 (S421) modulates early endosome motility by controlling switching between microtubule and actin components of the cytoskeleton. Huntingtin acts as a scaffold that interacts with both motors and cargoes, enabling it to regulate kinesin- and dynein-dependent transport. Kinesins transport cargoes toward the cell periphery and dynein moves them toward the cell center. These motors act in teams to direct the cargo towards its intracellular destination. Post-translational modifications of huntingtin allow precise control of motor protein activity. Previous studies in neurons showed that huntingtin phosphorylation at S421 increased the motility of brain derived neurotrophic factor vesicles toward the cell periphery. To mimic phosphorylation while maintaining the endogenous stoichiometry of interactions with huntingtin-associated proteins, we used clustered regularly interspaced short palindromic repeats (CRISPR) gene editing to generate a HEK293T cell line with the mutation serine 421 to aspartate (S421D). Rab5-positive early endosomes in S421D huntingtin cells exhibit increased processivity toward both the microtubule plus- and minus-ends. We hypothesize that huntingtin phosphorylation is responsible for regulation of cytoskeletal switching of cargoes between actin and microtubules, where the Rab5 cargoes in S421D cells spend more time being transported along microtubules while a higher fraction of Rab5 cargoes move along actin in the wild-type cells. The effect of phosphorylation is cargo specific. Interestingly, early endosomes labelled with epidermal growth factor-coated quantum dots did not demonstrate a significant difference in motility between the S421D and wild-type huntingtin cells, suggesting a subpopulation of endosomes are less affected by huntingtin phosphorylation. Lysosome motility is not affected by the S421D mutation. Our results show that Rab5 cargoes have increased processivity and run length in the huntingtin S421D mutant in microtubule plus- and minus-end directed motility, consistent with an increased fraction of cargoes being transported along microtubules upon phosphorylation. This work suggests that phosphorylation of huntingtin at site S421 leads to cargo-specific transfer of Rab5 cargoes from actin to microtubules to increase processivity.

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Differential regulation and fine tuning of kinesin and dynein motility by microtubule-associated septin complexes
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Intracellular transport is driven by the microtubule (MT) motors kinesin and dynein which mediate plus-end and minus-end directed transport, respectively. MT motor activity and directionality is regulated by cargo and adaptor proteins, microtubule-associated proteins (MAPs) and MT post-translational modifications. Septins are a large family of GTP-binding proteins that oligomerize into higher order complexes, which associate with MTs. We recently discovered that the MT-associated septin 9 (SEPT9) impedes the motility of kinesin-1/KIF5C and enhances kinesin-3/KIF1A, and through this differential regulation promotes neuronal axon-dendrite membrane polarity (Karasmanis et al, Dev Cell 46:204,
Because septins assemble into hetero-oligomeric complexes, we hypothesized that different septin complexes may exert different effects on kinesin and dynein motors. Here, we explored the possibility of a septin code in the regulation of motor motility by comparing effects of the MT-associated SEPT2/6/7 and SEPT5/7/11 complexes to each other and SEPT9 using in vitro single molecule motility assays of kinesin-1/KIF5C, kinesin-3/KIF1A and the dynein-dynactin-BicD2 (DDB) motor complex. We show that microtubule-associated mCherry-SEPT2/SEPT6/SEPT7 (100 nM) and mCherry-SEPT5/SEPT7/SEPT11 (50 nM) impede the motility of kinesin-1/KIF5C in a similar manner to SEPT9; SEPT5/7/11 has a higher MT affinity than SEPT2/6/7. Strikingly, we found that the motility of kinesin-1/KIF1A is differentially modulated by SEPT9, SEPT2/6/7 and SEPT5/7/11. MT-associated SEPT2/6/7 decreased the binding, velocity and run-lengths of kinesin-3/KIF1A, which are all enhanced by SEPT9. In contrast, SEPT5/7/11 did not affect the binding and run-lengths of kinesin-3/KIF1A, but had a mild impact on velocity and pausing. Consistent with previous effects of MT-associated SEPT9, DDB velocity and run lengths were significantly decreased by SEPT2/6/7 (20 nM) and SEPT5/7/11 (10 nM). Notably, SEPT2/6/7 and SEPT5/7/11 were much more potent inhibitors of dynein motility than kinesin, completely blocking MT-DDB binding at concentrations over 50 nM. Although both SEPT2/6/7 (20-50 nM) and SEPT5/7/11 (10-50 nM) inhibited the MT landing rates of DDB, we found that SEPT5/7/11 (50 nM) enhanced the number of immotile DDB particles, which was indicative of a drastic decrease in DDB detachment. SEPT5/7/11 was similarly unique in impeding the detachment of immotile kinesin-1/KIF5 and kinesin-3/KIF1A motors. Taken together, these data demonstrate that septins impact the motility of kinesin-3/KIF1A differentially, and how MT-associated septins fine tune motor motility by modulating individual parameters such as motor detachment in a septin paralog and complex specific manner.

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The mitochondrial adaptor TRAK2 functionally links opposing kinesin and dynein motors

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Mitochondria are transported by kinesin and dynein motors, which move in opposite directions along microtubules. These motors are linked to mitochondria by TRAK adaptor proteins, but the mechanism by which TRAKs coordinate the functions of these opposing motors is unclear. Here, we investigate the regulation of kinesin-1 and dynactin by TRAK2. We demonstrate that kinesin-1 and dynactin can concurrently bind TRAK2 and that TRAK2 enhances the association between these opposing motor components. We use single molecule reconstitution assays to show that TRAK2 moves processively in both directions along microtubules. Depletion studies demonstrate that TRAK2 transport to the microtubule plus-end requires kinesin-1 whereas transport to the microtubule minus-end requires dynein and dynactin. Minus-end directed transport is augmented by LIS1, similar to other dynein adaptors. TRAK2 transport towards the microtubule minus-end also depends on the TRAK2 CC1 box, as mutations to this motif significantly inhibit dynein-mediated transport. However, the activities of kinesin-1 and dynein are not independent of each other, as knockdown of one motor reduces the frequency of runs initiated by the opposing motor, suggesting that the holo-motor complex binds more effectively to the microtubule. Together, these observations support a model in which TRAK2 functionally links kinesin-1 and dynein-dynactin by forming an interdependent motor complex that coordinately regulates the bidirectional trafficking of mitochondria.
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The mitochondrial cargo adaptor TRAK1 has overlapping binding sites for kinesin-1 and dynein
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Organelle transport is central to the organization and function of eukaryotic cells. Positioning of axonal mitochondria into target locations is critical for the physiology of neurons as they supply energy and calcium buffering capacity. Two microtubule motor proteins kinesin-1 (“kinesin”) and cytoplasmic dynein-1 (“dynein”) are the primary carriers for bi-directional transport of mitochondria in neurons. Kinesin is an anterograde motor, while dynein is a retrograde motor trafficking in the opposite direction. In order for mitochondria to recruit motors, the outer mitochondrial membrane protein Miro cooperates with TRAK1 and TRAK2 to scaffold kinesin and dynein, driving anterograde and retrograde mitochondrial transport. To understand how TRAK1 regulates both kinesin and dynein, we sought to utilize biochemical reconstitution to define the minimal region of TRAK1 sufficient for promoting motor activation. First, to map the binding sites of kinesin and dynein on TRAK1, we designed a series of TRAK1 fragments to determine if the fragment can pull down endogenous kinesin (KIF5B) and dynein from HEK293 cells. Our work confirmed that TRAK1’s N-terminal coiled-coil binds to both kinesin and dynein comprising amino acids 1-395 TRAK1(1-395)). Interestingly, a shorter construct containing 1-340 retained kinesin binding even though dynein binding was abolished. Next, we sought to determine whether TRAK1(1-395) is sufficient to bind and activate full-length kinesin (isoform KIF5B) in vitro. To measure association, we performed size exclusion chromatography where we found that TRAK1(1-395) stably associates with kinesin, forming a complex that co-eluted in fractions from size exclusion. Then, we determined whether fluorescently-labeled SNAP-TRAK1(1-395) is an activator of kinesin using TIRF. After incubating SNAP-TRAK1(1-395) with kinesin, we saw that SNAP-TRAK1(1-395) increased kinesin’s run length and velocity relative to kinesin alone. After determining that TRAK1(1-395) activates kinesin, we next determined whether TRAK1(1-395) functions as a dynein activator in vitro. Motility experiments revealed that TRAK1(1-395) induces robust processivity of dynein-dynactin confirming that TRAK1 is a dynein activator. In summary, we characterized the first cargo adaptor that has overlapping binding sites for kinesin-1 and dynein that is capable of activating both motors in vitro. This indicates that the N-terminus of TRAK1 mediates motor protein binding while the C-terminus of TRAK1 likely mediates mitochondrial cargo binding. Future work will help to determine how TRAK1 modulates kinesin vs. dynein recruitment and regulation when TRAK1 is bound to mitochondrial cargoes.

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Reconstitution of DDB-kinesin bidirectional transport in vitro
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Robust bidirectional transport in neurons requires the kinesin-1, 2 and 3 superfamilies, which move toward microtubule plus-ends, and cytoplasmic dynein, which moves toward microtubule minus-ends. In vivo, cargo motility includes frequent switching between diffusive, paused and processive states, as well as reversals in direction. While the motility parameters of single motors, such as stall force, rates of attachment and detachment, and force-velocity relationships, are becoming well-understood, it remains unclear how these characteristics influence the behavior of multiple motors working cooperatively or antagonistically to transport cargo. Here, we use a DNA scaffold to pair mammalian dynein-dynactin-
BicD2 (DDB) with members of the kinesin-1, 2 and 3 families to recreate bidirectional transport in vitro, and determine how the distinct motility properties of the neuronal kinesin families impact transport. We found that pairing kinesin-1 with DDB results in a nearly equal amount of DDB-dominated and kinesin-dominated transport, as well as long paused states, but interestingly, we do not observe many reversals in direction or diffusive episodes. To resolve this discrepancy, we plan to use high resolution tracking of a kinesin-1 head labeled with a gold nanoparticle to directly measure the detachment rate of kinesin-1 in this context.

Adaptor proteins for opposing motors interact simultaneously with nuclear pore protein Nup358

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Nup358 is a protein subunit of the nuclear pore complex that recruits the opposing microtubule motors kinesin-1 and dynein (via the dynein adaptor Bicaudal D2, BicD2) to the nuclear envelope. This pathway is important for positioning of the nucleus during early steps of mitotic spindle assembly and it is also essential for an important process in brain development. It is unknown whether dynein and kinesin-1 interact with Nup358 simultaneously or whether they compete. Here, we have reconstituted and characterized a minimal complex of kinesin-1 light chain 2 (KLC2) and Nup358. The proteins interact through a W-acidic motif in Nup358, which is highly conserved among vertebrates, but absent in insects. While Nup358 and KLC2 form predominantly monomers, their interaction results in formation of 2:2 complexes, and the W-acidic motif is required for the oligomerization. In active motor complexes, BicD2 and KLC2 each form dimers. Notably, we show that the dynein adaptor Bicaudal D2 and KLC2 interact simultaneously with Nup358, resulting in formation of 2:2:2 complexes. Mutation of the W-acidic motif results in formation of 1:1:1 complexes. This is further supported by our small angle X-ray scattering data of the complexes. Based on our data we propose that Nup358 recruits simultaneously one kinesin-1 motor and one dynein motor via BicD2 to the nucleus. We hypothesize that the binding sites are close enough to promote direct interactions between these motor recognition domains, which may be important for regulation of motility of these opposing motors. Our data provide important insights into a nuclear positioning pathway that is crucial for brain development and faithful chromosome segregation.

Kinetochore-Microtubule Interface

The Astrin-SKAP Complex Makes the Kinetochore-Microtubule Interface More Dynamic and Force-Responsive

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The kinetochore links chromosomes to spindle microtubules to drive chromosome segregation during cell division. To do so, its attachments to microtubules must be both dynamic and strong. While we know nearly all mammalian kinetochore proteins, how these give rise to dynamic and strong
microtubule attachments remains poorly understood. Here, we focus on the Astrin-SKAP complex, which localizes to bioriented kinetochore pairs and is essential to chromosome segregation, but whose mechanical role is unclear. Live imaging and quantitative analysis reveal that SKAP depletion dampens metaphase kinetochore movements, reduces sister kinetochore coordination and increases the distance between them. In turn, kinetochore laser ablation shows that without SKAP kinetochore-microtubules depolymerize slower as the sister kinetochore moves poleward, and that more force is needed to rescue them to polymerize. Thus, in contrast to previously described kinetochore proteins that increase the grip on microtubules under force, Astrin-SKAP makes the interface more dynamic and force-responsive. Together, our findings suggest a model where Astrin-SKAP “lubricates” bioriented, correct kinetochore-microtubule attachments to help preserve them.

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**Kinetochore-microtubule detachment to promote error correction is independent of depolymerization for powering poleward chromosome movement**

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Faithful chromosome segregation is essential for cell viability and relies upon the correct bioriented attachment of sister kinetochores to the plus ends of spindle microtubules. In human cells, chromosome poleward movement is driven, in part, by the depolymerization of kinetochore-bound microtubule plus ends. Also, errors in the orientation of kinetochore-microtubule (k-MT) attachments are corrected by the release and depolymerization of the kinetochore-bound microtubule plus end. This study aims to investigate if microtubule depolymerization associated with chromosome movement influences the efficiency of k-MT detachment necessary for error correction. Live imaging of mitotic cells was used to measure multiple parameters including: the rate of poleward microtubule flux, inter-centromere distance, pole-to-pole distance, velocity of poleward chromosome movement, and the detachment rate of k-MTs, in a variety of conditions. Here, we focus on a potential relationship between poleward chromosome motion and microtubule detachment from kinetochores. In untreated human U2OS cells, poleward chromosome velocity is equivalent in prometaphase and metaphase (1.94 μm/min and 1.97 μm/min, respectively), although k-MT attachment stability, measured as a read out from k-MT half-life, significantly increases from prometaphase to metaphase (2.31 min and 3.79 min, respectively). To explore this question further, we specifically manipulated the activity of proteins known to be involved in the regulation of k-MT dynamics and chromosome velocity. Overexpression of Kif18A reduced poleward chromosome velocity to 0.80 μm/min as previously shown with a slight, but not significant, reduction in k-MT attachment stability (t1/2 = 3.0 min) compared to untreated metaphase cells. Stimulation of MCAK activity using the compound UMK57 did not significantly alter k-MT attachment stability (t1/2 = 3.21 min) and decreased velocity to 1.55 μm/min. In cells overexpressing Kif18A, the addition of UMK57 displayed chromosomes that were virtually immobile despite the presence of robust k-fibers. The half-life of k-MTs under these conditions was 2.14 min and equivalent to prometaphase cells. Taken together, these data indicate that the regulation of k-MT detachment rates necessary for error correction and depolymerization for powering chromosome poleward movement can act independently. Moreover, there is an unexpected functional relationship between the activities of Kinesin-8 and Kinesin-13 families of proteins on chromosome movement.
**Kinetochoore microtubule-associated factors, Ska and Cdt1 coordinate for forming robust end-on kinetochore-microtubule attachments**

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Cdt1, a DNA replication licensing protein, is recruited to kinetochores during mitosis via the loop domain of the Ndc80 complex (Varma et al., Nat Cell Biol., 2012). More recently, we demonstrated that Cdt1 is a novel microtubule-associated protein (MAP) at kinetochores. Further, Aurora B kinase phosphorylation of Cdt1 was found to be critical for modulating the strength of Cdt1-microtubule binding (Agarwal et al., J Cell Biol., 2018). Besides Cdt1, the Ndc80 loop domain has also been shown to be important for recruiting another MAP complex, the Ska1 (Ska) complex, to kinetochores. In this study, we use Auxin-induced Degron (AID)-tagging of Cdt1 to verify its role during mitosis. Further, we demonstrate a direct interaction between the two above-mentioned kinetochore-localized MAPs, i.e. Cdt1 and the Ska complex, using multiple biochemical approaches. We carried out Bio-Layer Interferometry (BLI) studies using purified Cdt1 and the Ska complex to calculate the dissociation constant (kd) of this interaction to be ~ 3.5 um. Ska-Cdt1 interaction was also detected in mitotic cellular extracts and was found to be essential for proper recruitment of Cdt1 to kinetochores. Total internal reflection fluorescence microscopy (TIR-FM) experiments demonstrated that Ska strikingly augments microtubule-binding of Cdt1. These findings were substantiated by studies in HeLa cells, where Ska facilitated enhanced localization of Cdt1 on spindle microtubules, indicating a synergy between these two MAPs for microtubule-binding. Consistent with that, depletion of Ska resulted in reduced binding of Cdt1 to spindle microtubules. TIR-FM experiments also show that Cdt1 synergizes with the Ndc80 complex for binding to microtubules as proposed by our previous studies (Varma et al., Nat Cell Biol., 2012). Our data points to a model where the Ska complex binds to Cdt1 and facilitates its docking on to the Ndc80 complex at kinetochores to facilitate the formation of a tripartite Ndc80-Ska-Cdt1 complex. We are currently testing if this tripartite complex between Ndc80, Ska and Cdt1 is able to bind to microtubules better as compared to the individual components. We postulate that this tripartite complex formation may be necessary to generate robust end-on kinetochore-microtubule attachments in vivo to cope with the dynamic flux of spindle microtubule growth and shrinkage, and contributing to persistent coupling of kinetochores to dynamic microtubule ends during mitotic metaphase.

**The nucleoporin and SUMO E3 ligase NUP358/RANBP2 regulates the mitotic factor NuSAP1 at the kinetochore/microtubule interface**

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Protein conjugation with SUMO (Small Ubiquitin-like MOdifier) peptides modulates protein interactions and localization in many cellular dynamic processes, e.g. DNA replication and repair, gene transcription, nuclear organization and others. Growing evidence also implicate protein SUMOylation in control of
mitosis. Indeed, several factors acting in cycles of SUMOylation and deSUMOylation reside at kinetochores, including SUMO ligase enzymes (RANBP2 and members of PIAS family) and SUMO deconjugating enzymes (SENP proteins). RANBP2 is a large nucleoporin with SUMO E3 ligase and SUMO stabilizing activity. It has long been known that, after nuclear envelope breakdown and disassembly of nuclear pore complexes, RANBP2 localizes at microtubules and in part at kinetochores. By developing the Proximity Ligation Assay (PLA) technique, coupled with cell synchronization methods, we have characterized a highly regulated mechanism, involving the nuclear transport receptors Importin beta and CRM1, which timely tethers RANBP2 at kinetochores during mitosis. Therein, RANBP2 interacts with several proteins and modulates their function, including for example Topoisomerase II (alpha). Here, we report a new role of RANBP2 on NuSAP1 (Nucleolar and Spindle Associated Protein 1), a microtubule-regulatory protein implicated in microtubule/kinetochore interactions and spindle assembly. We have developed intramolecular PLA methods and demonstrate that NuSAP1 is SUMOylated in vivo in a RANBP2-dependent manner in mitotic cells. Imaging, biochemical analyses and functional assays show that RANBP2 modulates the stability of a NuSAP1 fraction that localizes at the microtubule plus ends and needed to stabilize the interactions of chromosome with the spindle microtubules before anaphase onset. Together these results indicate that RANBP2-mediated SUMOylation modulates the process of microtubule-kinetochore attachments, at least in part by regulating the localization and function of NuSAP1, with implications in the mechanisms regulating proper chromosome segregation.

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Load-bearing interactions between the Ndc80 and Dam1 complexes differ on growing and shortening microtubule tips

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Accurate chromosome segregation requires the kinetochore to stably attach to the dynamic tip of a spindle microtubule. The Ndc80 and Dam1 complexes are the two main microtubule binding components in the kinetochore. Interaction between the Ndc80 and Dam1 complexes enhance the load-bearing ability of the kinetochore. We previously found that three different proteins in the Dam1 complex each binds to a different site in the Ndc80 protein. Electron micrographs show that the Ndc80 complex bridges two Dam1 complex rings in vitro. Mutations in any of these three sites disrupt the ability of the Ndc80 complex to bridge two rings in vitro and disrupt proper Dam1 complex localization to the rest of the kinetochore in vivo. However, which interaction sites between the Ndc80 and Dam1 complexes play a role in load-bearing is still unknown. As part of error correction, Aurora B kinase disrupts the interaction between the Dam1 and Ndc80 complexes by phosphorylation at any of the Dam1 complex proteins that interact with the Ndc80 complex (named A\textsuperscript{Dam1p}, B\textsuperscript{Ask1p}, and C\textsuperscript{Spc34p}). I utilized the optical trap to measure the strength of the Ndc80 complex attachment to an assembling microtubule in the presence of Dam1 complex phosphorylated at any of the Dam1 complex proteins that interact with the Ndc80 complex (named A\textsuperscript{Dam1p}, B\textsuperscript{Ask1p}, and C\textsuperscript{Spc34p}). I utilized the optical trap to measure the strength of the Ndc80 complex attachment to an assembling microtubule in the presence of Dam1 complex phosphorylated at each site. Phosphorylation at either region A\textsuperscript{Dam1p} or B\textsuperscript{Ask1p}, inhibits the load-bearing interaction between the Ndc80 and Dam1 complexes on an assembling microtubule. Phosphorylation at region C\textsuperscript{Spc34p} did not cause a defect. To test whether the same regions in the Ndc80p play a role in establishing load-bearing interactions with the Dam1 complex, I have generated three lethal insertion mutations along the Ndc80 protein (A\textsuperscript{Ndc80p}, B\textsuperscript{Ndc80p}, and C\textsuperscript{Ndc80p}). The addition of an insertion mutation at region A\textsuperscript{Ndc80p} conferred a complete defect in the ability of the Ndc80 complex to form a load-bearing interaction with the Dam1 complex on an assembling microtubule, while an insertion mutation in region B\textsuperscript{Ndc80p} conferred a partial defect and an insertion
mutation in region \( C^{\text{Ndc80p}} \) did not confer any defect. I next tested the role of different regions of Ndc80p to form load-bearing interactions on disassembling microtubules in the presence of the Dam1 complex. I found that insertion mutations in either region \( B^{\text{Ndc80p}} \) or region \( C^{\text{Ndc80p}} \) led to a 12-15 fold increase in detachment rates compared to wild-type. In conclusion, region A forms a load-bearing interaction between the Ndc80 and Dam1 complexes on an assembling microtubule tip. I plan to test its load-bearing ability on a disassembling microtubule tip. Region B forms load-bearing interactions between the Ndc80 and Dam1 complexes on both assembling and disassembling microtubule tips. Interestingly, region C only forms a load-bearing interaction between the Ndc80 and Dam1 complexes on a disassembling microtubule tip.

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**Tension promotes kinetochore-microtubule release by Aurora B kinase**

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To ensure accurate chromosome segregation, interactions between kinetochores and microtubules are regulated by a combination of mechanics and biochemistry. Tension provides a signal to discriminate attachment errors from bi-oriented kinetochores with sisters correctly attached to opposite spindle poles. Biochemically, Aurora B kinase phosphorylates kinetochores to destabilize interactions with microtubules. To link mechanics and biochemistry, current models regard tension as an input signal to locally regulate Aurora B activity. Here we show that the outcome of kinetochore phosphorylation depends on tension. Using optogenetics to manipulate Aurora B at individual kinetochores, we find that kinase activity promotes microtubule release when tension is high. Conversely, when tension is low, Aurora B activity promotes depolymerization of kinetochore-microtubules while maintaining attachment. Thus, phosphorylation converts a catch-bond, in which tension stabilizes attachments, to a slip-bond that releases microtubules under tension. We propose that tension is a signal inducing distinct error-correction mechanisms, with release or depolymerization advantageous for typical errors characterized by high or low tension, respectively.

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**A Microtubule-end Mediated Phospho-Signalling Cascade Protects Mature Chromosome-Microtubule Attachments Independent of Biorientation**

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Chromosomal instability (CIN) is a hallmark of several pathologies and can arise from errors in the process of microtubules capturing and segregating chromosomes. Chromosome-microtubule attachment is mediated by the kinetochore, a large macromolecular machine. Each kinetochore must be attached to microtubules from one spindle pole and its sister kinetochore attached to microtubules from the opposing pole - a state called biorientation to ensure accurate chromosome segregation. Non-bioriented kinetochore pairs experience reduced microtubule-mediated pulling; these attachment errors are known to be released by Aurora-B kinase. However, non-bioriented/mono-oriented
kinetochores are also an early step of the biorientation process; how these are selectively sensed and protected is not clear. Here we present evidence for signalling cascades transduced by microtubule-end binding proteins that sense outer-kinetochore structure changes specific to mature kinetochore-microtubule attachments, and in addition stabilise these mature attachments. Through an siRNA-mediated screen for microtubule-end associated proteins (MAPs) involved in attachment regulation, we identified outerkinetochore MAPs that protect non-bioriented attachments independent of Aurora-B. By revealing how MAPs delivered by microtubule-ends can dynamically regulate phospho-signalling cascades at the outer-kinetochore, we report a previously unrecognised important role for microtubule-ends in stabilising non-bioriented chromosome-microtubule attachment and in turn preventing CIN.

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A narrow dynamic range of kinetochore protein phosphorylation supports mitotic fidelity

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Faithful chromosome segregation during mitosis requires the formation of bi-oriented attachments between microtubules and kinetochores. Errors in attachments between kinetochores and microtubules frequently occur early in mitosis and must be corrected before anaphase onset to prevent aneuploidy from occurring. Error correction relies upon the phosphorylation of kinetochore proteins by kinases such as Aurora A/B, Cdk1 and Plk1 to selectively reduce their affinity for microtubules. Kinetochore phosphorylation can be reversed by phosphatases from the PP1 and PP2A families. The levels of phosphorylation must be precisely tuned to allow for initial attachments to form between kinetochores and microtubules, yet also permit robust correction of errors. Therefore, the levels of phosphorylation must not be set at extremes. However, the actual levels at which kinetochores are phosphorylated (i.e. absolute phospho-occupancy) are unknown. Here, we measured the phospho-occupancy of the conserved kinetochore protein Hec1 by mass spectrometry in mitotic cells under four different conditions ranging from a complete absence of microtubules through intermediate stages that allow for some improper attachments, to mostly correct bi-oriented attachments. We were able to obtain phospho-occupancy data from the five known Aurora B kinase sites S15, S44, S49/T50, S55 and S69 as well as an uncharacterized Cdk1 site at T31. Surprisingly, this analysis revealed that Hec1 phospho-occupancy ranges from only approximately 50% in cells lacking microtubules to 20% in cells with bi-oriented attachments. To further explore the role of the Cdk1 site at T31, we generated an antibody against phosphorylated T31. This antibody revealed maximal levels of phosphorylation at kinetochores in cells at a late prometaphase stage of mitosis. The location of T31 within Hec1 and the pattern of temporal regulation suggested a role for Cdk1 phosphorylation of T31 in the correction of kinetochore-microtubule attachment errors. Consistent with this hypothesis, we observed an increase in phosphorylated T31 at pole arrested and misaligned kinetochores. Moreover, we observed Cyclin B1-GFP present specifically at laterally attached, misaligned kinetochores in mitotic cells. Lastly, cells unable to phosphorylate Hec1 at T31 display an increased rate of lagging chromosomes in anaphase, demonstrating a loss of error correction function. Therefore, our data suggest a role for Cdk1-Cyclin B1 at kinetochores not only in regulation of the spindle assembly checkpoint, but also in the correction of kinetochore-microtubule attachment errors.
Microenvironment in Stem Cell Fate

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Chromosomal Instability of Induced Pluripotent Stem Cells in Culture
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Since their creation in 2007, induced pluripotent stem cells (iPSCs) have offered great promise in the field of regenerative medicine. By reprogramming a patient’s own cells, immunological rejection can be avoided during transplantation. Though iPSCs have much promise, they are still fraught with pitfalls which must be overcome, namely the accumulation of genetic aberrations that can occur in culture. During development and expansion iPSCs must be grown in artificial culture conditions for extend periods of time, which has the potential to introduce many types of genetic abnormalities. In addition to point mutations, these genomic alterations can range from aneuploidy to subchromosomal aberrations, characteristics often associated with cancer cells. To gain new insight into the nature of these chromosomal aberrations, iPSCs were grown and examined periodically over the course of 50 passages. While the iPSCs were being cultured, their genomic integrity was examined periodically using optical mapping technology. This technology can identify structural variations across the entire genome ranging from 500 base pairs to megabase pairs in length. Compared to next generation sequencing, optical mapping produces longer read lengths capable of better detecting large structural variants. Notably, inversions and balanced translocations, which do not produce copy number changes, can be distinguished. Optical mapping of iPSCs detected hundreds of structural variations, comprised of insertions, deletions, duplications, and inversions, not present in the general population. Multiple gene groups, such as those involved in cell differentiation and chromosomal rearrangement, were disproportionately affected by coding sequence variation caused by the detected structural variants. Also analyzed was the colocalization of structural variations with repetitious elements in the genome, which may enhance certain types of structural variation. In addition to subchromosomal changes, a third copy of chromosome 12 was detected in one line. This trisomy was confirmed via chromosome spreads, which indicated that the aberration entered the cell population at approximately passage 22 and became the dominant genotype within 3 additional passages. These studies draw new light on the potential danger of genome instabilities that evolve during culture of iPSCs. Supported by a grant from The Oklahoma Center for Adult Stem Cell Research to GJG and grants 5R35GM126980 to GJG and 1R01GM121703 to CLS from the National Institute of General Medical Sciences

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Matrix viscoelasticity regulates human pluripotent stem cell morphogenesis
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Human pluripotent stem cell (hPSC) derived organoids have tremendous potential for use in regenerative medicine and to model development in-vitro but are limited by their ability to mimic in-vivo morphology. Morphogenetic processes underlie human development and organoid formation, often occurring in 3D viscoelastic microenvironments. This includes the formation of embryonic tissues, which in humans commences at implantation, by a cluster of pluripotent stem cells. In response to
extracellular matrix signaling, these cells undergo polarization and lumen formation to give rise to the epiblast. Epiblast formation has been modeled in-vitro using culture of human induced pluripotent stem cells (hiPSCs) in 3D basement membrane-based matrices such as Matrigel. However, the role of matrix properties in directing hiPSC lumen formation remains unclear. While matrix properties have been shown to regulate morphogenetic processes, the impact of matrix viscoelasticity on stem cell morphogenesis is generally unknown. In this work, we use viscoelastic alginate hydrogels with independently tunable stress relaxation (a measure of matrix viscoelasticity), RGD ligand density and stiffness and show that matrix mechanics regulate hiPSC morphogenesis. Higher RGD density and fast stress relaxation promote hiPSC viability, proliferation, lumen formation and apicobasal polarization while slow stress relaxation at low RGD density triggers hiPSC apoptosis. Surprisingly, matrix stiffness did not significantly impact hiPSC viability, proliferation, apoptosis, pluripotency or lumen formation. hiPSCs in all alginate formulations maintained pluripotency through at least day 14 of culture. This is in sharp contrast with previously reported 3D Matrigel culture where hiPSCs form lumens but lose pluripotency after day 3 of culture. We find that lumen formation is regulated by actomyosin contractility as well as Rac1 activity and is accompanied by translocation of YAP from the nucleus to the cytoplasm. Modulating matrix stress relaxation provides control over lumen size and cluster characteristics of hiPSCs in fast relaxing, high RGD gels recapitulate key features of epiblast nuclear and cluster morphology. Our results reveal matrix viscoelasticity as a new factor regulating stem cell morphogenesis and provide key insights into the role of mechanical cues during development of the human embryo. These suggest that matrix viscoelasticity may be leveraged to build organoids mimicking in-vivo form and function.

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The Nuclear Envelope Regulates Neuroectoderm Lineage Specification During Exit From Naïve Pluripotency

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Cells have the remarkable ability to sense the biochemical and physical properties of their environment to inform their decision-making regarding differentiation. During their short existence in-utero, pluripotent stem cells gain competence to differentiate and make lineage choices as they exit naive pluripotency. Early differentiation of pluripotent cells occurs during implantation, where the pluripotent cells of the epiblast (or fertilized egg) undergo morphological change as they transition from a disordered ball of cells to an ordered and polarized epithelium. Notably, genome reorganization occurs during this process, and there is mounting evidence that proteins of the nuclear envelope (NE) could regulate this reorganization, although the significance of these NE proteins during exit from naïve pluripotency is still unclear. Nuclear envelope proteins are thought to generally bind to suppressed regions of the genome, while the nuclear envelope, via the LINC complex, is tethered to the cytoskeleton, which serves as a mechanical conduit to the physical and mechanical environment of the cell. It is still an open question whether physical cues in the cells’ environment can impinge on NE or LINC complex proteins to regulate early cell fate decisions during pluripotent stem cell differentiation through control of genome organization. We used an in-vitro mouse embryonic stem cell defined culture system that closely mimics pluripotent cells in-utero to investigate the role of NE proteins in regulating early cell fate decisions. We generated cell lines with inducible NE perturbations, including the ectopic
expression of Lamin-A to disrupt endogenous chromatin-NE interactions, and dominant-negative LINC mutants to decouple the LINC complex. Lamin-A expression and LINC decoupling did not impair exit from naïve pluripotency, but did impair the activation of the Sox1 neuroectoderm lineage gene. We then measured the localization and dynamics of the Sox1 gene locus during exit from naïve pluripotency using 3D DNA FISH and a novel, dCas9-based live genomic locus tracking method. This revealed that our NE perturbations altered the localization and dynamics of the Sox1 locus. These results suggest the importance of an intact linkage between the cytoskeleton, chromatin and NE in regulating neural-ectoderm lineage decision making. With these tools and additional specific NE and cytoskeletal perturbations, we aim to dissect the molecular mechanisms of genome regulation during lineage specification mediated by the nuclear envelope, cytoskeleton, and physical cues in the microenvironment.

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Differential Levels of the HSP70/HSP90-organizing protein STI1 Regulates Stemness and Proteostasis in Mouse Embryonic Stem Cells

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Stress-Inducible Protein 1 (STI1) is, among other physiological functions, an essential cochaperone for the formation of a functional complex between the heat shock proteins HSP70 and HSP90, playing crucial roles in protein homeostasis. Complete depletion of STI1 in mouse leads to early degeneration of the embryos, culminating in lethality. This demonstrates a key unexplored function of STI1 protein in initial stages of development. Pluripotent stem cells (PSCs), including mouse embryonic stem cells (mESCs), are frequently used as a model to study the early development. These cells have high rates of chaperones and cochaperones synthesis, which are critical to proteome integrity and proper function of many regulatory proteins involved in stemness. Therefore, mESCs expressing different levels of STI1 were used as a system model to investigate the role of this protein in pluripotency maintenance and its implications in molecular mechanisms involved in early development. Our results indicate that cells with decreased expression of STI1 have concomitant decrease in the expression of different pluripotency markers, such as alkaline phosphatase and the core transcription factors OCT4, SOX2 and NANOG. In addition, decreased levels of STI1 resulted in significant reduction in proliferation, and increased levels of DNA-damage and apoptosis markers. On the other hand, cells with higher STI1 levels show an enhanced expression of pluripotency factors and a substantial increase in proliferation rates, when compared to both wild-type and cells with reduced STI1 expression. A cell stress/DNA-damage protective effect is observed in STI1-overexpressed cells, since the levels of apoptosis and DNA-damage markers were reduced. Moreover, our data also demonstrates that STI1 may have an impact on the differentiation capacity of mESCs, since embryoid bodies expressing diminished STI1 levels have reduced diameter and volume. Together, these results suggest that STI1, a component of the proteostasis network, plays a fundamental role in pluripotency maintenance in mESCs. This work contributes to the still recent understanding of posttranslational control of pluripotency, helping to clarify possible central players, such as STI1 and its partners, as master’s post-genomic regulators of the pluripotent phenotype.
Raman measurement of exosomes obtained from cell culture supernatant of induced pluripotent stem cells

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Non-invasive automatic measurement methods for stem cell differentiation are critical for cell production in regenerative medicine applications. Exosomes, extracellular vesicles secreted by living cells, represents cellular constituents and properties of source cells, including proteins, nucleic acids, metabolites and lipids that have been considered ideal candidates for biomarkers. Here, we established a qualitative index based on the Raman measurement/spectra of exosomes in the culture supernatant of induced pluripotent stem (iPS) cells and iPS cell-derived neural lineage cells for the non-invasive evaluation of differentiation states of cultured cells. We employed Raman spectroscopy for quantitative analysis, which enables us to measure sample compositions without any preprocessing. Exosomes were extracted from the culture media of iPS cells (201B7) during differentiation (days 0, 8, and 12) to iPS cell-derived neural lineage cells using affinity purification targeting phosphatidylserine on exosome surfaces. Exosome fractions (n = 3) were measured using a home-assembled Raman spectroscope with the following parameters: excitation laser wavelength, 532 nm; laser source power, 100 mW; and scan time: 20 s. Primary component analysis of the Raman signals showed a significant clustering pattern among the three time points, suggesting a change in the exosomal components (proteins, nucleic acids, and lipids). Thus, we demonstrated the applicability of sequential Raman measurement in the non-invasive characterization of the differentiation state of iPS cells into neural lineage cells.

Structurally-discovered KLF4 Variants Accelerate and Stabilize Reprogramming to Pluripotency

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The cellular reprogramming technology is one of the most exciting achievements in life sciences; however, current stagnation of reprogramming technology accounts for such drawbacks as a poor and low yield of high-quality reprogrammed cells from primary somatic cells. Somatic cells can be inefficiently reprogrammed to pluripotent cells by exogenous expression of reprogramming factors,
which are mostly transcription factors. Transcription factors bind closed parts of the DNA to regulate downstream genes and to change epigenetic status toward reprogrammed cell states. Natural transcription factors have been used as reprogramming factors, but the functional alteration of reprogramming factors is unexploited overall. Low competence of natural factors may prevent the majority of cells to successfully and synchronously reprogram. Among reprogramming factors, Kruppel-like factor 4 (KLF4) is a pleiotropic protein that regulates various biological contexts, such as reprogramming, pluripotency, cancer, tissue development and homeostasis. Here, we tested whether modifications in amino acid residues of DNA-binding domains enable rational engineering of next-generation reprogramming factors. So, we screened amino acid residues in the zinc-finger domain of KLF4 for enhanced reprogramming efficiency. We demonstrated the proof of this concept by developing KLF4 variants, which accelerated and stabilized reprogramming to pluripotency in both mouse and human somatic cells, by mutant screening of DNA-interacting amino acid residues in KLF4’s DNA-binding zinc-finger domain. Identified KLF4 alanine substitution variant produced Nanog-GFP-positive mouse induced pluripotent stem cells (iPSCs) more rapidly and efficiently and also human iPSCs which were free from differentiation-defective iPSC markers. This KLF4 zinc-finger domain alanine substitution variant specifically bound more to promoters or enhancers of pluripotency genes, such as KLF5, and drove the gene expression of these genes during reprogramming. Moreover, molecular dynamics simulation analysis predicted that it formed a unique structural conformation with strengthened interactions with DNA. By iPSC generation experiments with all amino acid residue variants of exemplified alanine substitution position, we identified that this mutation position in zinc-finger domain represented a “molecular bump” to regulate structural stability of the interaction between zinc-finger domain and DNA, revealed by the findings that smaller amino acid residues showed higher reprogramming efficiency. Overall, our study demonstrates how modifications in amino acid residues of DNA-binding domains enable next-generation reprogramming technology with rationally engineered reprogramming factors.

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Insulin-like Growth Factor II is a Constituent in Adult Small Intestine Stem Cell Niche
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The insulin-like growth factors (IGFs) were first found in serum to promote skeletal muscle growth and cartilage sulphation, which was pituitary growth hormone (GH) dependent and in an “insulin-like” action. However, later studies identified their roles in cell growth were more prominent than those in metabolism. IGF-II is preferentially expressed in many somatic tissues during prenatal and neonatal growth and is much less GH dependent. Igf-2 knockout mice shown severe dwarf and growth retardation. In adult stage, IGF-II is mainly expressed in the liver and the epithelial cells lining the surface of the brain such as choroid plexus and leptomeninges and its local expression is essential for adult neurogenesis. Although its imprinting defect was identified with tissue overgrowth and a variety of tumors including colon cancer, its role in intestine stem cell (ISC) niche homeostasis is still unknown. Our work revealed that IGF-II mRNA and protein expression mainly in the intestinal epithelial cells (IEC), especially the crypt. Knockout of Igf-2 globally resulted in severe body weight drop and lethality, which
was due to a rapid loss of ISCs and dysfunction of the intestine. IGF-II expression was significantly decreased in knockout IECs, which resulted in malformed crypts and villi, significantly reduced number of stem, Paneth and transit amplifying cells at transcriptional level and protein level. Inducible deletion of Igf-2 in isolated organoids led to significantly increased number of dying organoids with reduced proliferative cells and disappeared budding formation. These dying organoids can be rescued by culturing with IGF-II ligand, indicating that the IGF-II secreted from epithelial cells is required for maintaining the crypts growth in vitro and the stem cell niche homeostasis in vivo. As the intestinal stem cell niche contains several autocrine/paracrine growth factors, our acute inducible deletion of Igf-2 may disturb the niche microenvironment, which could not be compensated in a short time. Thus, our findings unraveled the indispensable role of IGF-II in maintaining the adult stem cells not only of the neurons but also of the intestinal epithelial cells. This discovery contributed greatly to both intestine stem cell and the IGF society.

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**DNA damage promotes epithelial hyperplasia and fate mis-specification via fibroblast inflammasome activation**

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DNA crosslinking agents are widely used in cancer chemotherapy; however, responses of normal tissues to these agents have not been widely investigated. We reveal in mouse interfollicular epidermis, mammary gland, and hair follicle epithelia that genotoxicity does not promote apoptosis but paradoxically induces hyperplasia and fate specification defects in quiescent stem cells. DNA damage to skin causes epithelial and dermal hyperplasia, tissue expansion, and proliferation-independent formation of abnormal K14/K10 dual-positive suprabasal cells. Unexpectedly, this behavior is epithelial cell non-autonomous, and independent of an intact immune system. Instead, dermal fibroblasts are both necessary and sufficient to induce the epithelial response, which is mediated by activation of a fibroblast-specific NLRP3 inflammasome and subsequent IL-1β production. Thus, genotoxic agents that are used chemotherapeutically to promote cancer cell death can have the opposite effect on wild-type epithelia by inducing, via a non-autonomous IL-1β-driven mechanism, both hyperplasia and stem cell lineage defects.

**Microtubule Plus-End Dynamics**

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**Collective effects of XMAP215, EB1, CLASP2, and MCAK lead to robust microtubule treadmilling**

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Microtubule network remodeling is essential for fundamental cellular processes including cell division, differentiation, and motility. Microtubules are active biological polymers whose ends stochastically and independently switch between phases of growth and shrinkage. Microtubule treadmilling, in which the microtubule plus end grows while the minus end shrinks, is observed in cells; however, the underlying
mechanisms are not known. Here, we use a combination of computational and in vitro reconstitution approaches to determine the conditions leading to robust microtubule treadmilling. We find that microtubules polymerized from tubulin alone can treadmill, albeit with opposite directionality and order-of-magnitude slower rates than observed in cells. We then employ computational simulations to predict that the combinatorial effects of four microtubule-associated proteins (MAPs), namely EB1, XMAP215, CLASP2, and MCAK, can promote fast and sustained plus-end-leading treadmilling. Finally, we experimentally confirm the predictions of our computational model using a multi-MAP, in vitro microtubule dynamics assay to reconstitute robust plus-end-leading treadmilling, consistent with observations in cells. Our results demonstrate how microtubule dynamics can be modulated to achieve a dynamic balance between assembly and disassembly at opposite polymer ends, resulting in treadmilling over long periods of time. Overall, we show how the collective effects of multiple components give rise to complex microtubule behavior that may be used for global network remodeling in cells.

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The value of bent protofilaments at the ends of growing microtubules

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The ends of growing microtubules (MTs) are flared (McIntosh et al., J. Cell Biol. 2018; Gudimchuk et al., Nat. Commun. 2020). We have used electron cryo-tomography to study protofilament (PF) shapes under different conditions of growth, seeking insights into the pathways for tubulin polymerization and the physiological significance of these bent tubulin oligomers. 1) PF shapes are highly variable, both in cells and in vitro, but their average length is between 20 and 40 nm, depending on the conditions of growth and the species examined. Thus, they more than double the apparent diameter of each MT, increasing by more that four-fold the area each MT presents as it grows through the cell, seeking objects with which to interact. 2) The shapes of neighboring PFs are highly variable and show almost no correlation with one another, so they appear to be independent. Thus, each PF resembles a growing filament of FtsZ, a bacterial homolog of tubulin. This property indicates strong phylogenetic continuity in tubulin dynamics. 3) The ends of growing MTs are ragged in the sense that PFs peel out from the MT wall at different positions along the MT axis. The extent of raggedness is almost zero for shortening MTs, but for growing MTs, it increases with the speed of MT elongation. These properties may be one reason that flared PFs were evident in early electron microscopy of shortening MTs, but were not seen on growing MT until their ends were studied by electron tomography. 4) The speed of MT growth is increased both by TOG-domain proteins, which accelerate tubulin addition without increasing the average length of PFs, and by EB proteins, which bind the MT lattice near its growing tip. MT growth by flaring PFs rationalize the synergy of these controlling factors; when TOG proteins are present in quantity, and PFs are elongating quickly, then EB can catalyze more rapid formation of bonds between adjacent PFs as they straighten by thermal motions, further increasing the rate of MT growth. 5) Bent PFs on growing MTs provide a simple mechanism by which pulling forces can accelerate growth, explaining how oscillating metaphase chromosomes can retain spindle fiber attachments to the kinetochores on both chromatids, one of which is pulling while the other is being pulled.
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**XMAP215 promotes microtubule catastrophe in spite of an increased GTP-cap size**  
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The GTP-tubulin cap at the end of a growing microtubule is widely accepted to encode microtubule stability against catastrophe. The stabilizing GTP-cap can be visualized through microtubule end-localization of EB proteins, which recognize the nucleotide state of tubulin within the microtubule. When microtubule growth rate is increased in vitro by increasing tubulin concentration, the size of the ‘EB comet’ increases linearly, indicating that the GTP-cap size scales with the microtubule growth rate. At the same time, increasing tubulin concentration results in a lower catastrophe frequency, consistent with the hypothesis that catastrophe frequency negatively scales with the GTP-cap size. However, microtubule growth rates achieved with tubulin alone in vitro are an order of magnitude lower than those typically observed in cells. Fast microtubule growth rates can be reconstituted in vitro through synergistic effects of EB1 with XMAP215, a well-studied microtubule polymerase. How this combination of proteins impacts the GTP-cap size is unknown. Here, we observe that fast microtubule growth rate achieved using EB1 and XMAP215 in vitro correlates with an increased GTP-cap size. Interestingly, we show that the increase in GTP-cap size is accompanied by an increase in the catastrophe frequency, contrary to what is observed when microtubule growth rates are increased using tubulin alone. We perform growth-rate-matching experiments to directly compare the behavior of microtubules grown in the presence and absence of XMAP215. We find that microtubules polymerized with XMAP215 display a higher frequency of tapered microtubule end structures, in which a subset of protofilaments are growing faster than others. These microtubules also display a higher degree of growth fluctuations and brighter EB1 comets compared to microtubules grown without XMAP215. Our results underscore the role of the dynamically-evolving microtubule end structure in dictating overall microtubule stability.

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**Stochastic modeling of the interplay between microtubule catastrophes, tip structure and GTP hydrolysis**  
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Dynamic instability, a cycling between growing and shrinking periods, is a fundamental property of microtubules (MTs), which allows them to efficiently and continuously explore the intracellular space. The transition from growth to shrinkage, termed catastrophe, is a multi-step process, which has been suggested to depend on the configuration of the MT tip and hydrolysis of guanosine triphosphate (GTP) molecules, incorporated into the MT lattice. It has previously been widely accepted that GTP hydrolysis leads to a bending conformational change in tubulin dimer, the main MT building block. However, a growing body of structural data have revealed that tubulin dimers have curved equilibrium shapes, regardless of the phosphorylation state of their associated nucleotide. This important information urged us to revise existing models of MT dynamics, and MT catastrophe in particular. Here we develop a new
four-state Monte-Carlo model of MT dynamics, adding two conformational states (‘curved’ and ‘straight’) besides two nucleotide states of tubulin (‘GTP’ and ‘GDP’). The model treats lateral and longitudinal tubulin bond rupture as separate events responsible for either antagonizing tubulin bending or for keeping two dimers in a protofilament together, respectively. Thus, when calibrated, the model provides estimates of bond energies and dimer interface stiffness. Systematic analysis of this model’s behavior suggests an alternative way to associate conformational and configurational changes occurring at the MT end with the GTP molecule hydrolysis to improve our understanding of MT transition mechanisms. If a conventional ‘random-coupled’ GTP hydrolysis rule is assumed, our four-state model of MT dynamics cannot simultaneously recapitulate the increasing probability of MT catastrophe over MT lifetime and the GTP cap size, determined from tubulin dilution experiments. Introducing a tubulin conformation-dependent GTP hydrolysis substantially improves model performance with respect to its ability to describe a wide range of available experiments. Thus, we suggest, based on our modeling, that the current understanding of the GTP hydrolysis is oversimplified and should be re-visited in the context of the new view of MT polymerization with curved protofilaments at the tip. Supported by RF President’s grant MK-1869.2020.4.

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Microtubule Growth is Slowed Through Transient Exposure of GDP Tubulin

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Microtubule plus-end growth dynamics are determined by a kinetic race dictated by tubulin on- and off-rates at the microtubule tip. The apparent tubulin on- and off-rates at the plus-end can be estimated by measuring the mean plus-end growth rate across a range of free tubulin concentrations. However, when growth phases are studied at higher temporal resolution, fluctuations around the average growth rate are observed, implying more complex dynamics. To understand growth dynamics at the microtubule plus-end, we used Interference Reflection Microscopy (IRM) to quantify time-dependent variations in microtubule growth using GMPCPP, a non-hydrolyzable GTP analog. We found an expected linear dependence of the average growth rate on tubulin concentration. However, there was also a linear relationship between growth variance and tubulin concentration. Utilizing these measured values, we were able to constrain a biochemical model of microtubule growth with an on-rate of 1.1 / μM/pf/s. To determine the degree to which plus-end growth in GMPCPP mimics the GTP cap, we repeated the measurements in GTP. We again found a linear dependence of the average growth rate on tubulin; but found a much higher tubulin-dependent growth variance than expected from the GMPCPP experiments. When the growth variance was normalized to growth rate for each condition, there was a 5-fold increase in the relative growth variance in GTP compared to GMPCPP. This result suggests that GTP hydrolysis impacts tubulin binding kinetics at the microtubule plus-end. Consistent with this hypothesis, we then artificially varied the hydrolysis rates from 0 to 0.15 s⁻¹ in the biochemical model. Increasing the hydrolysis rate increased the growth variance, and transient suppression of growth coincided with increasing numbers of exposed GDP tubulin at the plus-end. This work suggests that during growth in GTP, exposure of terminal GDP tubulin subunits causes a slowing in microtubule growth until a terminal GTP is restored.
Microtubule dynamics and force production examined with multi-scale computational modeling and electron cryotomography

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Microtubules (MTs) are essential tubulin polymers that stochastically alternate between assembly and disassembly, exploring the intracellular volume and filling it with an array of transport tracks. MT dynamics can generate mechanical forces to affect cell shape, reposition organelles and segregate duplicated chromosomes during mitosis. Recently, we have shown that MTs grow by addition of curved guanosine triphosphate (GTP) tubulins to the tips of curved tubulin protofilaments in vivo and in vitro, challenging previous views about tubulin assembly, and yet establishing some continuity between crystallographic and biochemical studies of tubulin’s atomic structure and the pathway for this protein’s polymerization. Building on this structural finding, we now report results from a broad computational and experimental effort to understand this pathway for MT assembly and its implications for the cell biology of MTs. We have constructed a comprehensive model of MT dynamics and force generation, using three approaches at different scales: (1) all-atom molecular dynamics simulations to investigate effects of GTP-hydrolysis on the structure and stiffness of tubulin PFs; (2) Brownian dynamics simulations to link MT tip structure during assembly/disassembly with force production, (3) and Monte-Carlo simulations to examine MT transitions between growth and shrinkage on a longer time scale. Our simulations suggest that thermal fluctuations drive very frequent straightening of curved tubulin PFs to form lateral bonds and support MT elongation. High flexural rigidity of curved PFs does not limit MT growth rate, provided the lateral bonds are sufficiently strong. MT catastrophe is a complex process, which is affected by both GTP hydrolysis and configurations at the MT tip. GTP hydrolysis probably affects the flexibility of tubulin PFs, rendering tubulin inter-dimer interfaces more rigid. We identify the activation energy barrier in lateral tubulin-tubulin interactions as a key hidden parameter, controlling the magnitude of pulling forces that can be generated during MT disassembly. To test our model predictions, we have used cryoET to examine MT tip shapes under a range of conditions, including the presence of a polymerization catalyst and tubulin-binding drugs. These analyses elucidate potential mechanisms for the regulation of MT assembly by associated proteins and drugs. Finally, our simulations provide a mechanism for load-dependent acceleration of MT assembly, offering a role for flared MT tips in synchronizing MT growth and shortening rates during chromosome oscillations. Supported in part by RFBR grant # 20-34-70159 and NIH grant # GM33787.
**Mitochondrial Quality Control**

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**ALS-associated TBK1 mutations differentially disrupt mitophagy**

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TANK-binding kinase 1 (TBK1) is a multi-functional kinase with an essential role in mitophagy, the selective clearance of damaged mitochondria via autophagy. TBK1 translocates to depolarized mitochondria downstream of PINK1 activation and Parkin recruitment, and promotes autophagosomal engulfment. More than 90 TBK1 mutations are linked to the neurodegenerative disease amyotrophic lateral sclerosis (ALS), indicating a central role for TBK1 in maintaining cellular homeostasis; however the functional consequences of these mutations are unclear. We investigated how ALS-associated missense mutations of TBK1 affect Parkin-dependent mitophagy using live and fixed-cell imaging to dissect the molecular mechanisms involved in clearing mitochondria. Mutations that disrupt the ability of TBK1 to dimerize, associate with the mitophagy receptor optineurin (OPTN), auto-activate, or catalyze phosphorylation of target molecules have differential effects on mitophagy, with some mutations causing severe dysregulation of the pathway and others having minor effects. Namely, both TBK1 recruitment and OPTN phosphorylation are necessary for engulfment of the damaged mitochondrion by an autophagosomal membrane. The ALS-linked TBK1 mutations G217R and M559R disrupt both dimerization and auto-phosphorylation; we find these mutations inhibit the translocation of TBK1 to damaged mitochondria, thus preventing OPTN phosphorylation although OPTN recruitment remains unaffected. However, the R357Q mutation, which disrupts only dimerization, has no effect on OPTN phosphorylation, nor the proportion of mitochondria that are positive for the autophagosomal marker LC3, challenging the hypothesis that TBK1 must dimerize in order to carry out its function. Our study further refines the model for TBK1 function in mitophagy and demonstrates that some ALS-linked TBK1 mutations likely contribute to disease pathogenesis by inhibiting mitophagic flux. We also demonstrate that several TBK1 mutations have a less disruptive effect on mitophagy, suggesting other TBK1-dependent pathways such as innate immunity and inflammation may also contribute to the development of ALS. Supported by Project ALS (Grant ID 2018-03) and NINDS (NS060698).

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**Mitochondrial Proteostasis Requires Genes Encoded in a Neurodevelopmental Syndrome Locus**

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Eukaryotic cells maintain homeostatic balance between protein synthesis and degradation, or proteostasis, through mechanisms that require both cytoplasmic and mitochondrial translation. Genetic defects affecting cytoplasmic translation perturb synapse development and neurotransmission, and are causative of neurodevelopmental disorders such as Fragile X syndrome. In contrast, there is little indication that mitochondrial proteostasis is required for synapse development and function. Here, we focus on two genes deleted in 22q11.2 deletion syndrome, a recurrent copy number variation that is one of the strongest genetic risk factors for schizophrenia. We demonstrate that SLC25A1 and MRPL40, two genes present in the 22q11.2 microdeleted segment whose products localize to mitochondria, physically and genetically interact and are necessary for mitochondrial protein translation and proteostasis. Our data are the first to indicate that the citrate transporter SLC25A1 plays a role in mitochondrial translation and ribosome stability, and that SLC25A1 and mitochondrial ribosome subunit MRPL40 participate in the same pathway. Downregulation of these genes in *Drosophila* neurons affects morphology of the developing neuromuscular junction, alters neurotransmission, and disrupts sleep behavior. In addition, bioinformatic analyses of publicly available data reveal differential regulation of human mitochondrial ribosome subunit gene expression by brain region, cell type, and developmental time period. Expression of mitochondrial ribosome genes is also altered in post-mortem brains from patients with schizophrenia. Together, our data suggest that mitochondrial ribosomes are required for synapse development and function and influence behavior. We propose that disruptions to mitochondrial proteostasis, either by genetic or environmental factors, are a novel pathogenic mechanism contributing to neurodevelopmental disorders.

**P421**

**Mitochondria-associated degradation pathway (MAD) function beyond the outer membrane**

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The mitochondria-associated degradation pathway (MAD) mediates ubiquitination and degradation of mitochondrial outer membrane (MOM) proteins by the proteasome. We find that the MAD, but not other quality control pathways including macroautophagy, mitophagy, or mitochondrial chaperones and proteases, is critical for yeast cellular fitness under conditions of chronic, low-level oxidative stress in mitochondria produced by treatment with paraquat (PQ). Specifically, inhibition of the MAD increases PQ-induced defects in growth and mitochondrial quality and decreases chronological lifespan. We used mass spectrometry analysis to identify possible MAD substrates as mitochondrial proteins that exhibit increased ubiquitination in response to PQ treatment and inhibition of the MAD. We identified candidate substrates in the mitochondrial matrix and inner membrane and confirmed that two matrix proteins are MAD substrates. Our studies reveal a broader function for the MAD in mitochondrial protein surveillance beyond the MOM and a major role for the MAD in cellular and mitochondrial fitness in a model for aging based on mitochondrial oxidative stress.
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Role for Polarity Machinery in Lifespan Through Effects on Mitochondrial Quality Control During Inheritance in the Budding Yeast *Saccharomyces cerevisiae*

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Babies are born young, largely independent of the age of their mothers, in all organisms studied including single-cell eukaryotes like budding yeast. Our previous studies revealed that mitochondria are asymmetrically inherited during yeast cell division and that inheritance of higher functioning mitochondria by daughter cells and retention of some high functioning mitochondria in mother cells are critical for cell fitness, lifespan and mother-daughter age asymmetry. We found that the mitochondrial F-box protein, Mfb1p, contributes to lifespan control by mediating anchorage and retention of higher-functioning mitochondria to sites of cell polarity in a cell cycle-regulated manner: it localizes to and anchors mitochondria to the mother cell tip throughout the cell cycle, and to the tip of the bud at the end of the cell cycle. Here, we report that yeast cell polarity and polarized localization of Mfb1p decline with age in *S. cerevisiae*. Moreover, we find that deletion of *BUD1/RSR1*, a Ras family protein required for cytoskeletal polarization in response to cortical cues during asymmetric cell division in yeast, results in depolarized Mfb1p localization, defects in mitochondrial retention at the distal tip of the mother cell, compromised mitochondrial quality, and reduced lifespan. Finally, the double mutant of *mfb1Δbud1Δ* demonstrates no additive effects compared to either single mutant in mitochondrial distribution, mitochondrial quality or lifespan. Thus, we obtained evidence that *BUD1* and *MFB1* are in the same pathway to control the healthspan and lifespan of yeast cells. Taken together, our results demonstrate a new role of polarity machinery in lifespan control through modulating Mfb1 function in asymmetric inheritance of mitochondria during yeast cell division.

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The cell cycle-related kinase AURKA forms a tripartite complex with PHB2 and LC3 to select metabolically-competent mitochondria by mitophagy

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AURKA is a serine/threonine kinase with multiple functions during interphase and cell division. It is frequently overexpressed in epithelial cancers, correlating with poor patient survival and resistance to therapies. Recent evidence showed that AURKA localizes and activates at mitochondria. At this location, AURKA regulates mitochondrial dynamics, and its overexpression boosts mitochondrial ATP levels. However, the way AURKA orchestrates its different mitochondrial functions and interacts with its over 200 mitochondrial partners remains elusive. While exploring how AURKA interacts with the inner mitochondrial membrane (IMM) protein Prohibitin 2/PHB2 - one of the main interactors of the kinase at mitochondria ∼, we observed a partial loss of the mitochondrial pool upon the overexpression of AURKA in human cells. Quantitative microscopy and complementary biochemical approaches *in cellulo* and *in vivo* revealed that this mitochondrial loss recapitulates several features of mitochondrial elimination by
mitophagy, i.e. the proteasome-dependent loss of Outer Mitochondrial Membrane (OMM) proteins and the autophagy-dependent degradation of IMM/matrix content. We also determined that such mitochondrial loss is independent of the PINK1/Parkin pathway, but it is PHB2-dependent. In addition, Förster’s Resonance Energy Transfer (FRET) revealed that AURKA phosphorylates PHB2 on Ser39. This modification is needed to accommodate the mitophagy mediator LC3 on PHB2 itself, where it simultaneously interacts both with PHB2 and AURKA. This identifies a “AURKA-PHB2-LC3” tripartite complex triggering mitophagy upon AURKA overexpression. Altering the formation of the tripartite complex using the PHB2 ligand Xanthohumol or the AURKA inhibitor MLN8237 impaired mitophagy and restored normal ATP levels. Our results show a novel mechanism by which overexpressed AURKA, a cancer-like paradigm, triggers mitophagy. When overexpressed, AURKA forms a tripartite complex with PHB2 and LC3 to dispose of metabolically-inefficient mitochondria while preserving the organelles producing high ATP quantities. This mechanism could be a selective advantage for cancers cells to overcome cell death. Blocking the AURKA-PHB2-LC3 tripartite complex - and thereby blocking mitophagy - could represent a promising strategy to lower mitochondrial ATP levels in patients with cancer linked to AURKA overexpression.

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Quality control during inheritance of the mitochondrial genome

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Mitochondria are often referred as the powerhouse of the cell, because they supply virtually all eukaryotic cells with energy in the form of ATP. The respiratory chain is responsible for ATP-synthesis and is embedded in the mitochondrial inner membrane. Mitochondria contain their own genome, the mitochondrial DNA (mtDNA), which is present inside the mitochondrial matrix. Multiple copies of mtDNA are distributed along the mitochondrial network. In the baker's yeast S. cerevisiae, the 85 kb long mtDNA encodes for eight genes. Seven of those encode essential subunits of the respiratory chain and one encodes a protein of the small subunit of the mitochondrial ribosome. Mutations in mtDNA can lead to an impairment of the respiratory chain and therefore to a shortage in cellular ATP supply. Therefore, it is of utmost importance for cellular health that the integrity of mtDNA is maintained. We asked whether the single-celled yeast S. cerevisiae can distinguish between mutant and intact mtDNA and support generation of daughter cells with a healthy mtDNA content. To this end, we developed an assay to track the inheritance of either healthy or mutated mtDNA in single cells. Heteroplasmic cells were generated by mating a strain containing wildtype mtDNA with a strain harboring a deletion in the mtDNA encoded COB gene. Transmission of mutant or wildtype mtDNA from the resulting zygote was then monitored in single cell lineages for up to six generations. This analysis revealed that yeast cells predominantly produce daughter cells containing intact mtDNA. Intriguingly, selection still occurred in the absence of mitochondrial fission, which is surprising because such cells contain only one interconnected mitochondrial network. Interestingly, we found that mutants lacking genes required for cristae biogenesis lose the ability to select between healthy or defective mtDNA. This finding points to an important role of the mitochondrial ultrastructure in the maintenance of mtDNA integrity. We are currently aiming to understand how cristae support selection of wildtype mtDNA over mutated mtDNA.
Neuronal Degeneration and Regeneration

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**Increased LRRK2 kinase activity alters neuronal autophagy by disrupting the axonal transport of autophagosomes.**

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Parkinson’s disease-causing mutations in the LRRK2 gene hyperactivate LRRK2 kinase activity and lead to increased phosphorylation of Rab proteins, master regulators of intracellular trafficking. We found that the most frequent LRRK2 mutation, LRRK2-G2019S, dramatically decreased the processivity of axonal autophagosome transport in neurons in a kinase-dependent manner. This effect was consistent across multiple models, including LRRK2-G2019S overexpressing rat hippocampal neurons, cortical neurons from a G2019S knock-in (KI) mouse, and gene-edited human iPSC-derived G2019S KI neurons. In contrast to the observed effects on autophagosome transport, LRRK2-G2019S did not affect the motility of LAMP1-positive late endosomes/lysosomes nor did the mutation perturb microtubule dynamics. Interestingly, we found Rab29, a known activator of LRRK2, to be associated with axonal autophagosomes. LRRK2 hyperactivation induced by Rab29 overexpression decreased the processivity of autophagosome transport to a similar extent as expression of the G2019S mutation in LRRK2. Downstream of hyperactive LRRK2 activity, our data indicate there is enhanced recruitment of the motor adaptor protein JIP4, which induces abnormal activation of kinesin-1. This aberrant activation results in a tug-of-war between anterograde and retrograde motors, and thus disrupts the normal pattern of highly processive retrograde autophagosome motility along the axon. Disruption of autophagosome transport correlated with a defect in autophagosome acidification, suggesting that the observed transport deficit impairs effective degradation of autophagosomal cargo. Our work robustly links increased LRRK2 activity to defects in autophagosome transport and maturation in neurons, further implicating defective autophagy in the pathogenesis of Parkinson’s disease.

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**Investigating the spatiotemporal dynamics of OPTN-mediated mitophagy in neurons**

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Damaged mitochondria are selectively removed from the cell in a process termed mitophagy. This quality control mechanism may be important for neuronal homeostasis, as mutations of proteins involved in this pathway cause Parkinson’s disease and ALS. We used live imaging to gain mechanistic insight into the spatiotemporal dynamics of mitophagy in primary neurons. To probe the time-course of neuronal mitophagy, we developed a pulse-labeling paradigm to identify mitochondrial populations based on ‘age’. We find that mild oxidative stress induces low levels of mitochondrial damage and mitophagy, without compromising the entire neuronal network. Mitophagy-associated proteins, including Parkin, TBK1, and OPTN, rapidly translocate to depolarized mitochondria and damaged organelles were efficiently sequestered in autophagosomes within an hour of damage. We readily observed these mitophagic events in somatodendritic compartments, suggesting neuronal mitophagy is primarily a somal quality control pathway. Surprisingly, the downstream degradation of damaged mitochondria is remarkably slow. The acidification to fully turnover sequestered mitochondria was rate-
limiting and occurred hours to days after initial damage. Similar experiments were performed in non-neuronal cells, where we visualized rapid acidification of depolarized mitochondria an hour after insult. Thus, the time-course of mitophagy is slower in neurons. Expression of an ALS-associated mutation in OPTN was sufficient to disrupt mitochondrial network function and mild oxidative stress exacerbated this effect. These results suggest that slow turnover of damaged mitochondria may increase neuronal susceptibility to neurodegeneration.

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**Ediditary Spastic Paraplegia-SPG4is caused by gain-of-function cytotoxicity and exacerbated by SPG4-haploinsufficiency**

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Mutations of the SPAST gene are the predominant cause of Hereditary Spastic Paraplegia (HSP). SPAST encodes spastin, a microtubule-severing protein with membrane-associated properties. Controversy exists as to whether SPAST-based HSP (termed HSP-SPG4) is caused by haploinsufficiency or cytotoxic gain-of-function properties of mutant spastin proteins. Strong support for the latter has recently emerged from a new mouse model (termed SPAST-C448Y mouse) that has both endogenous SPAST alleles intact but also expresses human spastin bearing a pathological mutation identified in patients with HSP. Unlike SPAST-knockout mice, which show only very mild motor deficits and no corticospinal dying-back degeneration, SPAST-C448Y mice show adult-onset gait deficiencies and corticospinal dying-back degeneration similar to human patients. However, SPAST-knockout mice, like human patients, display pathological swellings of their corticospinal axons, while SPAST-C448Y mice do not. Here we crossed SPAST-C448Y mice with SPAST-knockout mice and found that the double transgenic mice display more severe gait deficiencies (as evaluated by the Catwalk assay) and more severe corticospinal dying-back degeneration than the SPAST-C448Y mice. In addition, the corticospinal axons of the double transgenic displayed swellings like the knockout mice and the human patients. These results support a model for SPAST-based HSP in which gain-of-function cytotoxicity of mutant spastins is necessary and sufficient for the disease but that haploinsufficiency is an exacerbating factor that renders axons more vulnerable to the toxicity of the mutant spastins. To further understand the mechanisms of the disease, isogenic human induced pluripotent stem cells (hiPSCs) harboring the C448Y (missense) and S245X (truncation) mutations and also SPAST-KO were established. Consistent with observations on the mice, the SPAST-KO shows axonal swellings but no dying-back degeneration, whereas the cells with mutant spastin genes showed both swellings and dying-back degeneration. Elevated activity of histone deacetylase 6 (HDAC6) identified in the mutants, but not in the KO, suggested a potential gain-of-function pathological pathway leading to the disease. Reduced microtubule mobility in the KO and mutant lines suggested a potential mechanism underlying the axonal vulnerability that exacerbates the HSP phenotypes. Overall, the present studies provide novel mechanistic information on how gain-of-function cytotoxicity and haploinsufficiency each contribute to the disease, which is important toward the development of an innovative toolbox of combinatorial therapies.
Microtubule-based mechanisms and therapies for Gulf War Illness using hiPSC-derived neurons and organoids

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Gulf War Illness (GWI) is a chronic disorder suffered by at least 30% of the nearly 700,000 U.S. veterans of the 1991 Gulf War. Central nervous system (CNS) symptoms include chronic fatigue, reduced information processing speeds, and memory deficits, among others. Evidence suggests that GWI is caused by a combination of the stress of the battlefield and exposure to organophosphate pesticides and nerve agents at repeated low doses, thus implicating novel biological targets. We hypothesize that many of the cellular changes contributing to the CNS symptoms of GWI are due to defects in microtubule-related processes, which can be partially corrected by microtubule-based therapies. Here, we used human-induced pluripotent stem cells (hiPSCs) derived from veterans of the 1991 Gulf War, some with and some without GWI. The hiPSCs were differentiated into glutamatergic neurons or forebrain cerebral organoids and then exposed to a GWI toxicant regimen of cortisol plus low-dose Diisopropyl fluorophosphate (DFP), a sarin analog. The cortisol+DFP toxicant regimen increased the levels of total tau and early pathologically phosphorylated tau, and reduced microtubule acetylation, a marker of microtubule stability. This toxicant regimen also reduced glutamatergic neuronal firing rates, bursting rates, and network synchrony, indicating reduced neuronal activity at the individual cell and circuit levels. Furthermore, some of these cellular changes were worse in cells derived from veterans with GWI compared to cells derived from GW controls, suggesting underlying vulnerabilities in veterans who developed GWI. In forebrain cerebral organoids, the cortisol+DFP toxicant regimen also increased astrocyte reactivity and impaired neurogenesis. These results indicate that a relatively mild toxicant regimen of cortisol plus low-dose DFP can be used to elucidate early, highly sensitive cellular changes in GWI. Our next step is to ascertain whether these defects can be corrected by microtubule-based therapies, mainly focusing on restoring microtubule acetylation to normal, which we previously showed rescued cellular defects such as mitochondrial transport in primary rat embryonic neurons. We will also use an adult rat model to ascertain whether these cellular defects, potentially correctable by microtubule-based therapies, contribute to GWI behavioral symptoms such as memory deficits.

Role of related MAP triple kinases DLK and LZK in the vincristine-induced neuronal injury response in human neurons

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Dual leucine zipper kinase (DLK; MAP3K12) and leucine zipper kinase (LZK; MAP3K13) are key mediators of the neuronal injury response shared among neurodegenerative conditions such as amyotrophic lateral sclerosis (ALS) and chemotherapy-induced neuropathic pain (CIPN). These related MAP3 kinases initiate a transcriptional cascade resulting in phosphorylation of the transcription factor c-Jun, facilitating the fate of neuronal cell death. Previous studies show that, while DLK disruption is partially protective in models of optic nerve injury and traumatic brain injury, combined disruption of DLK and
LZK is ultimately required to prevent retinal ganglion cell death (Welsbie et al, 2017 and 2019). Using human iPSC-derived cortical and sensory neurons, we sought to tease apart the distinctive and synergistic roles of DLK and LZK in the transcriptional response to neuronal injury. Single knockout iPSC lines of DLK and LZK, as well as a DLK/LZK double knockout, were generated using CRISPR/Cas9. Vincristine, a chemotherapeutic agent known to cause CIPN and activate the DLK/LZK pathway, induces partial c-Jun activation in both DLK KO and LZK KO neurons, but this activation is blocked in DLK/LZK double KO neurons. Transcriptomics in these cell lines will elucidate the relative contributions of DLK and LZK to the vincristine-induced neuronal injury response and potentially reveal new drug targets for neurodegenerative pathologies.

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Pathogenic Role of Delta 2 Tubulin in Bortezomib Induced Peripheral Neuropathy

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Chemotherapy-induced peripheral neuropathy (CIPN) is a debilitating “dying back” neuropathy featuring a distal-to-proximal peripheral nerve degeneration seen in cancer patients undergoing chemotherapy. Modification of the chemotherapy treatment of a patient is sometimes required to limit the severity of CIPN, preventing patients from receiving effective cancer treatment. The pathogenesis of CIPN is largely unknown, and this incomplete knowledge is a main reason for the absence of effective neuroprotection strategies while maintaining chemotherapy drug anticancer activities. Several classes of anticancer drugs with different antineoplastic mechanisms can induce CIPN. However, sensory impairment is the predominant adverse effect associated with each class, suggesting the existence of a common mechanism of pathogenesis. Tubulin and microtubules (MTs) are well-established targets for multiple anticancer drugs that can also induce CIPN. The contribution of the MT changes to the onset of CIPN is not well understood but is strongly implicated as a determining factor. Here, we report that the CIPN-causing drug, bortezomib, a proteosome inhibitor with anti-tumor activity in haematological malignancies, promotes the accumulation of delta 2 tubulin (D2), an irreversible tubulin post-translational modification residing on hyperstable MTs, while affecting microtubule stability and dynamics in sensory neurons in vitro and in vivo. The accumulation of D2, is predominant in unmyelinated fibers and a hallmark of bortezomib-induced peripheral neuropathy in humans. Furthermore, while D2 overexpression was sufficient to cause axonopathy and inhibit mitochondria motility, reduction of D2
levels alleviated both axonal degeneration and the loss of mitochondria motility induced by bortezomib. Together, our data demonstrate that bortezomib, a compound structurally unrelated to tubulin poisons, affects the tubulin cytoskeleton in sensory neurons in vitro, in vivo and in human tissue, indicating that the pathogenic mechanisms of seemingly unrelated CIPN drugs may converge on tubulin damage. These findings reveal a previously unrecognized role for the disruption of the tubulin tyrosination/detyrosination cycle in the onset of axonal injury that may occur through D2-dependent regulation of mitochondria dynamics.

New Techniques in Cell Biology: CRISPR

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Increased donor DNA sequence integration by CRISPR/Cas9 in mice: mechanistic insights
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Although the percent mice with CRISPR produced indels can easily reach 100%, the percent mice having a desired point mutation when a ssDNA donor is added is typically much lower, particularly if the point mutation is far from the Cas9 generated cut. Our recent results (doi: 10.3390/genes11060628) suggest that we can greatly increase the rate of successful point mutation integration in two ways. One requires first selecting a donor complementary to the guide’s variable region and ending near the guide’s 5’ end, not the 3’ end. The donor, guide, and Cas9 should then be injected into the zygote in phase G2. The second way, meant for incorporating a point mutation far from the cut, is to introduce mismatches about every 10 base pairs in the donor between the cut site and the desired point mutation. Our results showed that when a donor was complementary to the guide, integration of the donor mutations usually occurred either in all cells of the mouse blastocyst with cut DNA or in none of them. The simplest explanation is that the blastocysts with integrated mutations are primarily only those where injection happened after replication of the zygote pronuclear DNA i.e. during G2. This suggests that homology-directed repair may be far more favorable than non-homologous end joining during G2 if the donor has already begun repair by binding to and elongating on the free strand (doi: 10.1038/nbt.3481) produced after a Cas9 cut. We also showed that by using donors with mismatches roughly every 10 base pairs between the cut and desired mutation, we completely eliminated the lower rate of sequence incorporation normally seen for these mutations when far from the cut. We think the resected genomic DNA end searching on the side of the cut with the mismatches, is not able to bind well or at all to this region, and so can’t elongate. On the other hand, the region in the donor ending beyond the desired point mutation will have a competitive advantage because when it invades the genomic DNA region beyond this last mutation, it matches perfectly. Hence, it can bind and extend, thus incorporating the donor’s mutational sequence. For practical use, the mismatches can be selected so as to produce silent mutations in the genomic DNA. In conclusion, we have detailed some novel mechanisms for more efficient donor sequence integration into the mouse genome when using CRISPR.
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**CRISPR Toolkits for 3D Genome Research**  

The CRISPR-Cas technology provides a powerful toolkit for genome editing and gene regulation. Beyond editing, we developed a CRISPR-mediated toolkit to manipulate, image, and study the 3D genome structure, function and dynamics. The toolkit includes a CRISPR-Genome Organization (CRISPR-GO) platform for programmable control of spatial genome organization, and a CRISPR live-cell fluorescence in situ hybridization (LiveFISH) platform for DNA and RNA imaging. The CRISPR-GO system allows one to efficiently manipulate the spatial positioning of desired genomic locus relative to specific nuclear compartments in the nucleus, including the nuclear periphery, Cajal bodies, and promyelocytic leukemia (PML) bodies. Genomic positioning via CRISPR-GO is inducible and reversible, and can be programmed to flexibly target different genomic sequences. Targeting specific genomic loci to the nuclear periphery or Cajal bodies repressed gene expression. The CRISPR LiveFISH system is built on an intrinsic stability switch of CRISPR guide RNAs, which uses conditionally stable fluorescent oligonucleotides for DNA and RNA tracking in diverse cell types, including primary patient cells. LiveFISH allows for accurate detection of chromosomal disorders such as Patau syndrome in prenatal amniotic fluid cells and tracking multiple loci in T lymphocytes. In addition, LiveFISH allows tracking the real-time movement of DNA double-strand breaks induced by CRISPR-Cas9-mediated editing and consequent endogenous chromosome translocation. Finally, combining Cas9 and Cas13 systems, LiveFISH allows for simultaneous visualization of genomic DNA and RNA transcripts in living cells. Thus, the CRISPR LiveFISH enables real-time imaging of DNA and RNA dynamics during genome editing, transcription, and rearrangements in single cells.

CaTCH - A barcode-guided CRISPRa-inducible reporter to isolate clones from heterogeneous populations  
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The emergence of resistant cell clones to targeted therapies poses a significant issue in the treatment of metastatic melanoma. While these founding clones are often extremely rare in a starting population, their isolation and characterization holds unique potential for understanding disease processes, uncovering novel biomarkers and developing therapeutic concepts. The functional characterization of such founder clones and comprehensive comparisons to their post-selection counterparts requires live cells. To achieve this, we developed a novel lineage tracing tool termed CaTCH (CRISPRa tracing of clones in heterogeneous cell populations). CaTCH combines precise mapping of the lineage history of millions of cells with the ability to isolate any given clone alive from a complex population based on genetic barcodes. CaTCH thereby enables the retrospective isolation and analysis of founding clones from heterogeneous cell populations prior to evolutionary selection. In first applications, we use CaTCH to provide insights into the development of resistance to targeted cancer therapies. We demonstrate
that CaTCH can be used to trace and isolate a single pre-existing therapy-resistant clone from a complex cancer cell population in vitro. Furthermore, we validate the utility of CaTCH for applications in vivo by investigating the origins of resistance to clinically relevant RAF/MEK inhibition in an immunocompetent melanoma mouse model. Here we find that most clones have the capacity to acquire resistance to combined RAF/MEK inhibitor therapy, indicating that resistance to this clinically relevant regimen is a universally achievable state in this model. We envision that CaTCH will address fundamental questions in basic and translational research (e.g., how cell identity states and trajectories are determined in therapy resistance, metastasis formation, tissue development and somatic cell re-programming), potentially revealing new vulnerabilities that can serve as targets for therapies.

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In vivo Perturb-seq: studying gene function in developing tissues
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The thousands of disease risk genes and loci identified through human genetic studies far outstrip our current capacity to systematically study their functions. I will discuss our attempt to develop a scalable genetic screen approach, in vivo Perturb-Seq, and apply this method to the functional evaluation of 35 autism spectrum disorder (ASD) de novo loss-of-function risk genes. Using CRISPR-Cas9, we introduced frameshift mutations in these risk genes in pools, within the developing brain in utero, and then performed single-cell RNA-Seq in the postnatal brain. We identified recurrent and cell type-specific gene signatures from both neuronal and glial cell classes that are affected by genetic perturbations. We validated our results in orthogonal systems and experiments: our data suggests a role of an ASD risk gene Ank2 in the Ndnf+ interneuron subtype during cortical development, in addition to its known roles in excitatory neurons for axonal morphology, connectivity, and calcium signaling. Moreover, we identified abnormal oligodendrocyte development upon the perturbation of Chd8 and validated this result in germline mutant mice. Lastly, many key genes and cell types that are altered in ASD patient brains, e.g. SST in interneuron and RAB33A in excitatory neurons, also showed similar changes in the Perturb-seq data, across a panel of different ASD risk genes. Altogether, these results pointed at elements of both convergent and divergent cellular effects across many ASD risk genes. In vivo Perturb-Seq pilots a systems genetics approach to investigate at scale how diverse mutations affect cell types and states in the developing brain, and can be used in various in vivo and in vitro model organisms and systems.

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Corallochytrium limacisporum, a newly emerging model system for comparative cell biology among the Holozoa clade
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The Holozoa clade emerges as an important group for comparative cell biology analyses among eukaryotes. In addition to the well-studied Metazoa group, it includes four different unicellular lineages. Interestingly, these lineages, are highly heterogeneous with diverse cell morphologies, developmental modes, life stages and importantly, can transiently form multicellular structures that evoke the initial steps of coordinated and specialized cell behaviour. The best way to understand in depth their biology is by performing functional analysis. To do so we need to have genetic tools in each of these lineages. To have the complete functional platform for comparative cell biology we need to establish genetic tools in the remaining lineage of Corallochytrea. C. limacisporum, belonging to Corallochytrea is a marine free-living walled saprotroph, that in addition to its key phylogenetic position, has a well-annotated genome containing several homolog genes with animals, and can be easily cultured in axenic conditions, in liquid and solid medium facilitating the isolation of clonal lines. The life cycle of C. limacisporum has still not been characterized. We have developed a stable transfection in C. limacisporum along with a battery of cassettes tagging key cellular components, nucleus, plasma membrane, cytoplasm and actin filaments. These tools allowed for the live observation of cells with labelled structures, and have already served for a better understanding of the life cycle of C. limacisporum revealing some characteristic features, which are rarely found in holozoans. We discovered two different paths for cell division, binary fission and coenocytic growth, demonstrating that C. limacisporum life cycle is non-linear. Unveiling the factors involved in the decision making of which developmental path is taken will significantly contribute not only to a better understanding of the mechanisms of cell division, but also to the understanding of the origin of simple multicellularity in unicellular Holozoa. Additionally, we found that C. limacisporum is binucleated for the majority of its life cycle. Interestingly, unlike most studied eukaryotes, the nuclear division is decoupled from the cellular division. We could also identify the exact number of nuclei in coenocytic cells discovering that nuclei can be divided in an asynchronous manner resulting in intermediate stages with odd numbers of nuclei. In this study, C. limacisporum has been developed into a experimentally tractable organism that will contribute in the understanding of key questions on the evolution of cellular mechanisms towards multicellularity, in the decision making of different developmental routes, in the decoupling of karyokinesis from cytokinesis, and the basis of asynchronous division.

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Evolutionarily conserved mechanisms governing branched morphology in the aggregatively multicellular Rhizarian amoeba, Filoreta ramosa

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Evolutionary mechanisms of cell organization and multicellularity involve emergent morphological complexity to generate spatial differentiation. While multicellularity has evolved multiple times across the eukaryotic supergroups, the Rhizaria, a clade including numerous multicellular amoebae, remains understudied. Our recently isolated Rhizarian amoeba, Filoreta ramosa, exhibits “aggregative multicellularity.” Individual amoebae migrate together and fuse to form a multinucleate syncytial network that develops a complex reticulated pattern using pseudopodial branches. Through mechanisms reminiscent of neuronal growth cones and dendritic arborization, the syncytium can grow
to span several centimeters in diameter. Nuclei and organelles undergo rapid and bidirectional long-range transport along microtubules within these branches at rates averaging 4.8 µm/sec. Fragmented branches are capable of self-recognition and auto-fusion, facilitating rapid repair, expansion, and dynamic interactions with the environment. How and when did the cytoskeletal mechanisms that drive polarized growth cones and branching morphology evolve in Eukarya? We are using our recently completed Filoreta genome combined with super-resolution imaging and morphometric analyses to quantify conserved cytoskeletal elements of syncytial morphology and development. We raised antibodies specifically to Filoreta γ-TURC components and EB1 to visualize non-centrosomal microtubule nucleation and polarity in proliferating pseudopodia. We compared morphological perturbations following treatment with cytoskeletal drugs affecting microtubule and actin dynamics. As in neuronal branches, the network is organized with longitudinal microtubule arrays coupled with actin-rich pseudopodial protrusions to initiate branch formation. Cytoskeletal drugs alter network development, underscoring the critical role of microtubule and actin dynamics and their interactions in generating the complex branched morphology in Rhizaria and Metazoa. We predict that the mechanisms governing the intricate cytoskeletal networks in Filoreta are emergent properties of simple branch and anastomosis patterns involving conserved cytoskeletal proteins. Thus, the branching mechanisms typified by neuronal outgrowth are conserved in Rhizaria and pre-date the divergence of major eukaryotic lineages.

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**Novel regulation of fructose 1,6-bisphosphatase in the protozoan parasite Trypanosoma brucei**

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Fructose 1,6-bisphosphatase (FBPase) is a key enzyme in the metabolic process gluconeogenesis (GNG), which synthesizes the glucose 6-phosphate necessary to fuel glycolysis and the pentose phosphate pathway. In eukaryotes, GNG and glycolysis are typically localized together in the cytoplasm and coordinated via allosteric regulation of the enzymes FBPase and phosphofructokinase (PFK). The protozoan parasite, *T. brucei*, cycles between the glucose-rich (~5mM) mammalian bloodstream and the tsetse fly where glucose levels are typically low. While in the bloodstream, parasites rely on glycolysis for ATP production while parasites in the tsetse fly primarily use oxidative phosphorylation and the role of GNG is unclear. FBPase was identified in the *T. brucei* genome in 2003 and recent metabolic labeling experiments revealed that GNG is active in bloodstream form and insect stage parasites. FBPase activity has been difficult to monitor in cell cultures, likely due to the low expression of the protein. We have designed a sensitive *in vitro* coupled enzyme assay to measure FBPase activity in cell lysates and determined how extracellular glucose and culture density influence FBPase activity and protein expression in insect-stage parasites. In stationary phase, cells grown in low-glucose have high levels of FBPase activity while cells in high-glucose have low levels of FBPase activity. In contrast, in log phase cells FBPase activity is highest in high-glucose conditions where GNG is expected to be inactive and undetectable in low-glucose where GNG is predicted to be active. Western analysis revealed FBPase abundance did not change with culture density indicating that protein levels are not responsible for these differences in activity. Our observations suggest that FBPase is not exclusively involved in GNG and that the enzyme may play an additional role in parasite metabolism. As we identify proteins and pathways that are affected by changes in FBPase expression and activity levels we anticipate identification of unexpected roles for FBPase in the protozoan parasite.
The Galactosyltransferase Domain of Glucosylceramide Synthase Regulates the Cyst Production and Morphology of *Giardia* by Modulating its Cellular Lipid Homeostasis

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The early diverging protist, *Giardia lamblia*, colonizes the human small intestine and causes a zoonotic diarrheal illness called "giardiasis." *Giardia* cysts, which transmit the infection, are made of water-resistant fibrous cyst walls containing glycans and proteins. Our laboratory has reported earlier that glucosylceramide transerase or synthase (gGlcT1), an enzyme of the sphingolipid (SL) metabolic pathway, acts as a key regulator of cyst production (encystation) in *Giardia*. gGlcT1 is an ER membrane protein and expressed upon encystation. Surprisingly, we found that gGlcT1 catalyzes the synthesis of both glucosylceramide (GlcCer) and galactosylceramide (GalCer) with the help of two separate catalytic domains: ceramide glucosyltransferase (CGlcT) and ceramide galactosyltransferase (CGalT). Using three different gGlcT1 constructs (endo-gGlcT1-HA; α2-Tub-gGlcT1-AU1 and OCT-gGlcT1-HA), we monitored the expressions and reaction profiles of CGlcT and CGalT and elucidated their roles in encystation and cyst production. It was observed that while the overexpression plasmid α2-Tub-gGlcT1 triggered the expression of CGalT activity in encysting cells, overexpression with OCT-gGlcT1 preferentially stimulated the CGlcT activity in trophozoites and encysting cells. On the contrary, wild-type cells induced both domains (CGlcT and CGalT) in encysting cells. Furthermore, the cysts produced by the wild type (Endo-gGlcT1-HA) and OCT-gGlcT1-HA appeared to be type I (regular morphology). On the contrary, cells carrying α2 Tub-gGlcT1 plasmid produced type-II cysts with irregular morphologies and poorly formed cyst walls. Cysts counts were also lower in α2-Tub-gGlcT1-AU1. These results indicate that the regulated expression of CGalT in encysting cells is important for cyst formation and its overexpression produced defective (type-II) cysts. Next, we carried out a high-resolution lipidomic analysis by UHPLC-MS/MS. We observed that the lipid profiles (in both negative- and positive-ion modes) in the α2-Tub-gGlcT1 clone were dramatically different than wild-type and OCT-gGlcT1 cells, suggesting that cyst lipids were affected by irregular CGalT activity and could be associated with altered cyst morphology and viability. More recently, we have generated CGlcT- and CGalT- domain specific knockdowns of gGlcT1 and are testing the role of each domain in cyst production, morphology, viability and the global lipidome.

A Kelch-domain containing nuclear protein PfMORC modulates the synchronization by melatonin in asexual stage of the human malaria parasite *P. falciparum*

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Malaria is one of the deadliest diseases in the world, accountable for the death of more than 400,000 people across the continents in tropical and sub-tropical regions. The responsible pathogens belong to the *Plasmodium* spp. of apicomplexan protozoa and among them; *P. falciparum* is most virulent with the highest fatalities. *Plasmodium* parasites switch between vertebrate host and mosquito vector to complete it’s asexual and sexual life-cycle respectively. The clinical symptoms of malaria are associated with the periodic rupture and exponential increase of parasites in the host’s circulation system. The endogenous hormone plays a significant role in modulating parasite progression in both vector and the
host. The host hormone melatonin has been reported to synchronize the asexual cell cycle of the human malaria parasite *P. falciparum*. Moreover, melatonin up-regulates a subset of genes involved in the ubiquitin-proteasome system (UPS) thus indicating a complex and multi-tasking signaling cascade for hormone action in *P. falciparum*. The leading factors regulating the parasite cell cycle are poorly understood. Using a real-time assay, we provide evidence of the expression of a kelch domain-containing protein (PfMORC) in the parasite’s asexual cycle. The expression of PfMORC has a negative effect in an orphan protein kinase 7 knockout (PfPK7−) parasites following melatonin treatment, which we have shown previously as a non-responsive to melatonin. Parasites expressing 3D7morc-GFP indicate nuclear localization of the protein during the asexual stage of development. We also developed glucosamine inducible knockdown of PfMORC which had no significant difference in parasite proliferation in culture but appreciably changed the ratio of different asexual intraerythrocytic stages of the parasites upon addition of melatonin. Our data reveal the potential role of PfMORC during intraerythrocytic parasite development and also indicates the complex signaling mechanism linked with the host hormone melatonin. **Key Words:** Plasmodium, Melatonin, Cell-cycle, Kelch-containing protein

**Scholarship of Diversity**

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**Influences to Notions of Diversity For Women of Color STEM Majors in the Community College**

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**Objective:** This case study aims to center the perspectives of women of color STEM majors in the community college and determine the influences to their notions of diversity of ideas and people in the STEM fields. **Methods:** Ego-centric social network analysis was performed via a name generator social network questionnaire that was answered by 36 women of color STEM majors in a two-year Hispanic-Serving Institution (HSI) in an urban, metropolitan area in Southern California. One of the questions in the survey was “Can you think of individuals who have influenced your notions of diversity of ideas or of people in STEM settings?” Participants (N = 36) provided the names of any influences and demographic characteristics of influences, including relationship to participants. Eight primary relationships emerged from the nominations: (1) family, (2) college faculty and staff, (3) K-12 educators, (4) friends (met outside of college campus), (5) college schoolmates, (6) local community members (outside of family, educators, friends, and schoolmates), (7) public figures (such as famous scientists like Albert Einstein), and (8) religious figures. Normalized degree centralities were calculated by obtaining the percentage of nominations for each relationship group per participant and then adding the percentage units in a summative total then percentized out of summative total nominations. **Results:** Findings showed that participants nominated their schoolmates as the most impactful group on their notions of thinking about diversity, with normalized degree centrality at 33.33% of all nominations. Following schoolmates in normalized degree centrality, college faculty and staff obtained 22.62% while family earned 19.94% of all nominations. **Conclusion:** In conclusion, peers such as schoolmates have a substantial impact on how women of color community college STEM students think about diversity in the STEM fields. This infers the significance of creating a campus and STEM classroom environment with diverse bodies and opportunities to engage in meaningful interactions.
Inclusive Excellence: How to build capacity for inclusion of all students and dismantle barriers to workforce diversity

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The dynamic demographics of the US population present a historic and compelling opportunity for institutions of higher education to transform STEM workforce diversity. All students, from all backgrounds and all interests, should have a meaningful and positive experience in science through which they will better understand and engage in scientific thinking and discovery. Faculty and administrators are responsible for the quality of every student’s experience and their respective institution’s culture. Achieving an inclusive science education culture across the higher education landscape requires a transformative approach that provides a brave space for learning, promotes student belonging, and cultivates an expectation of student success. The HHMI Inclusive Excellence initiative (IE) aims to create a learning community of colleges and universities who are engaged in the continuing process of increasing their institution’s capacity for inclusion of all students through reflection, sharing, and learning. The IE community currently includes HHMI and 57 U.S. colleges and universities. The 57 colleges and universities are a diverse mixture of public and private institution types, from baccalaureate colleges to doctoral universities. Fourteen of these schools serve significant numbers of students from groups historically excluded in higher education. IE community participants, who are faculty, staff, administrators, and students, have multiple opportunities to reflect on what they are learning, share their learning with others, engage in professional development activities, and explore areas of interest to support their work. This takes place through (i) annual self-assessments using the Progress towards Inclusive Excellence through Reflection (PIER), (ii) annual meetings of the entire IE community; (iii) on-line and in-person convenings of the Peer Implementation Clusters (PICs) in which each PIC comprises 4-5 IE institutions; and (iv) professional development activities organized by the PICs. The PIER gives grantees an opportunity each year to communicate and share their reflections on their ongoing lessons learned and where necessary, recalibrate approaches. The HHMI IE initiative provides five years of funding support, serving as a catalyst for change at institutions to assess their context and build capacity for inclusion and equity for all students in science. Through a partnership with the Inclusive Excellence Commission appointed by the American Association of Colleges and Universities (AAC&U), we are learning how colleges and universities can achieve culture change with respect to inclusion and better understand how to support institutional change in a rapidly evolving academic landscape.

Awareness and expressions of systemic racism in science: Impacts of the Inclusion and Social Mindfulness in STEM (ISMs) workshops on faculty and staff

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Despite a slow rate of change in faculty and staff demographics, strategic plans at departmental and institutional levels across the United States call for increases in the diversity of the student body. In light of this mismatch, we aimed to increase understanding of and action against racial bias and systemic
racism at a mid-sized, regional, primarily-undergraduate university. We created a four-part series of professional and personal development workshops designed to guide conversations and enable actions around issues of race in Science, Technology, Engineering, and Math (STEM). The Inclusion and Social Mindfulness in STEM (ISMs) workshops include four sections: (1) Cultural Awareness of self, (2) Experiences of others, (3) Critical Conversations, (4) A Call to Action. We assessed the impacts of the ISMs workshops on faculty and staff in two ways: 1) a set of written pre-post surveys; 2) participants were interviewed in focus groups. The majority of participants self-identified as white. ISMs participants reported increased awareness of topics that are explicitly covered in the workshops: intersectionality, microaggressions, and marginalized groups. Participants reported that their knowledge surrounding topics that are not covered explicitly in the workshops did not increase. Critically, there was no significant change in participants’ reported confidence in their own use of language, though there was a significant increase participants’ likelihood to “avoid using language that reinforces negative stereotypes” and “make efforts to educate yourself about other groups”. The thematic analysis of focus group videos of workshop participants revealed entrenched expressions of an ignorance and discomfort in discussing whiteness by the participants. This appeared to result in participants wanting to distance themselves from the responsibility to confront racism in the academy. Psychological distancing also figured into participants’ negative perceptions of colleagues who did not participate in the workshops. Bolstering themselves in this way may have shifted the focus away from participants’ own responsibility to confront structural racism. Overall, faculty and staff who participated in the ISMs workshops were more able to identify some elements of race and racism in their experiences in and out of the classroom but were often not aware of how whiteness and white culture affected their roles as teachers, researchers, and community members.

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Scientific societies join forces to amplify effectiveness of STEM diversity, equity, and inclusion efforts

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Scientific societies aiming to foster inclusion of scientists from underrepresented (UR) backgrounds among their membership often delegate primary responsibility for this goal to a diversity-focused committee. The National Science Foundation has funded the creation of the Alliance to Catalyze Change for Equity in STEM Success (ACCESS), a meta-organization bringing together representatives from several such STEM society committees to serve as a hub for a growing Community of Practice. Its goal is to coordinate efforts to advance inclusive practices by sharing experiences and making synergistic discoveries about what works. As examples of our work, we compare and contrast the ways in which scientific societies implement/assess their travel award programs for UR scientists as well as approaches by which member societies have sought to ensure inclusivity through selection of annual meeting
speakers. We discuss how inclusive practices in these two areas foster better scientific environments for all. We also identify challenges and promising practices for societies striving to maximize inclusivity of their travel award programming and diverse speaker representation at their annual meetings.

Science Education: Teaching in the Virtual World

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Creative Tension in Molecular Cell Biology Teaching through Covid-19
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During the Covid-19 pandemic and the transition from in-person to virtual class and lab teaching, I share reflections on Parker Palmer’s concept of creative tension in teaching, and how it has shaped the validation of students’ voices during virtual community of scholars team presentations. In addition, through each moment, I reflect and share of how lives of scientists connect with the career pathways of undergraduates. These undergraduates include first-generation Native American, Latinx, African-Americans, and non-traditional pathways in both virtual class and undergraduate research experiences in virtual environments. Finally, I will share not only initial qualitative analysis, but also how Covid-19 pandemic has shaped the concept of advocating in the moment to support undergraduates during times of uncertainty during the Covid-19 pandemic.

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Effect of Switching Cell Biology from Partially Flipped to Fully Online on Student Engagement and Performance
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Due to the COVID-19 pandemic, instruction in Cell Biology switched mid-semester, from partially flipped to fully online. The purpose of this study is to determine how exam grades and student engagement with online videos were affected by the change in instructional methods. A survey was also completed by students to determine whether or not access to the internet was a barrier to their learning and their perceptions of how their time and effort were affected by the change in course delivery. Analysis of student engagement with online lecture videos posted in the LMS before and after the change allows comparison of student viewing patterns. Two exams were given before and two exams after the change in instruction, allowing a comparison of exam grades before and after the switch. Initial analysis shows that exam averages before and after the switch were not statistically different, and were similar to averages from previously reported spring semesters. Sixteen percent of students reported problems with internet that prevented full engagement with the course, but this resulted in limited changes in exam grades for students without reliable internet. About half of the class indicated that they had a difficult time with the transition to online learning that was unrelated to internet access. This analysis will help inform future plans for online course delivery.
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**Pandemic pedagogy: lessons from a virtual experience**

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Teaching virtually during the COVID-19 pandemic facilitated assessment of student participation and achievement before and after the switch to a virtual environment. Penn State University, as did most universities in the United States, decided to switch to a virtual environment following Spring Break in March 2020. We examined student participation and grades in three courses: Cell Biology II, Organic Chemistry II, and Developmental Neurobiology. We compared student grades on the last two exams in each course as well as student participation in the courses. In all three courses, participation was unaffected by the switch to remote learning. Students continued to attend virtual class and continued doing work, even when learning from home. The grades on the exams, one of which was the final exam, were not statistically different from the previous two semesters. In thinking about the success we realized during the remote phase of the semester, one of the lessons we learned was that when faced with the prospect of teaching remotely (through ZOOM) the main focus was how to best encourage student engagement with the content. Students in an online environment still need to interact with content in meaningful ways that will help them learn information and retain that information long-term. We learned that a well-designed course can be adapted to alternative course modality. While course delivery has value, the role of the teacher as delivery conduit is sometimes over emphasized. The pandemic seems to have shifted the focus to design. The authors have been teaching blended and online courses for more than a decade and have witnessed greater comfortable level of students who learn remotely. During the pandemic students seemed capable of adjusting to the online format. A lesson for designing future courses seems to involve flexibility. Our main conclusion seems to be that well-designed courses can better serve to serve the changing needs of learners. Course design has been overlooked for too long as a critical aspect of effective teaching. The pandemic has helped spotlight the importance of well-constructed, organized courses that allow students to learn either in class or virtually.

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**Outcomes of classroom vs. remote instruction for undergraduate science classes in the backdrop of a global pandemic**

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With the recent necessity for teaching from a distance during the COVID-19 pandemic, undergraduate science faculty have been compelled to teach lecture and laboratory content through remote platforms. The purpose of this study was to assess outcomes in test performance, student participation, and retention in distance versus face-to-face undergraduate Biology courses. Two courses were assessed: Introductory Biology-I (BIO-I) and Anatomy & Physiology-II (A&P-II). In summer 2020, each course was conducted through a synchronous video platform using course structure comparable to traditional face-to-face classes. Laboratory content was kept as similar as possible to hands-on labs using a combination of simulations and at-home activities. Test results, class participation, and class retention were compared with the same courses taught face-to-face in the previous summer. In Bio-I, tests were delivered online asynchronously with restricted time limits. In A&P-II, tests were also delivered online;
however, all students took exams simultaneously. Test averages in BIO-I and A&P-II were consistent with test averages in the previous summer. The retention rate in BIO-1 was higher compared with the 2019 retention rate for this class. This was likely due to several factors, such as increased student visits to office hours and increase in diversity of the student population. In contrast, the retention rate in A&P-II was lower compared with the 2019 retention rate. In this class, few students took advantage of office hours. For both BIO-I and A&P-II, participation was similar with participation in summer 2019. In addition, students in both classes who consistently attended sessions with video enabled performed better compared with students who did not engage with video. Student feedback indicated that video classes provided a real class setting that benefited them. In conclusion, while face-to-face instruction is the preferred method for undergraduate science classes, remote synchronous classes might benefit student learning if students can be encouraged to participate.

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Analysis of CURE - generated data supports its utility for supporting faculty
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Incorporating Course-based Undergraduate Research Experiences (CUREs) into the classroom can provide numerous benefits to both students and faculty. While the student benefits have been well researched, few studies have investigated the faculty benefits. The purpose of this study was to provide insight on faculty benefits of CUREs by investigating the quality and usefulness of CURE-generated data. Five years of student-generated data from the Austin College Cancer Biology CURE was collected and analyzed to evaluate its consistency and applicability. The Cancer Biology CURE asked students to investigate a gene and its role in early carcinogenesis by performing karyotyping, transwell migration assays, wound-healing assays, and genotyping. Overall, analyzed data demonstrated high variability, but they tended to follow the general trends observed in published literature. As such, while the student-generated data may not meet the standards for peer-reviewed publication, they can inform projects that may be pursued outside of the course in the faculty member’s research lab. To date, 7 independent research projects at Austin College have been informed by Cancer Biology CURE-generated data. Additionally, the CURE-generated data have provided an opportunity for the faculty member to gain more publicity and awareness for their project through a total of 64 student presentations. Beyond faculty benefits, this study confirmed the CURE was providing the expected student benefits. Student development was analyzed by using student’s survey responses and end-of-course faculty assessments. These assessments focused on five different skill categories: communicating ideas, collaborative work, problem solving, foresight and planning and acting responsibly. With these results, we compared skill development in students who were enrolled in the CURE to the students in all STEM related courses. The results demonstrated that junior and senior biology majors in Cancer Biology developed skills through the course, consistent with expectations for their level of education and course experience. Overall, this study suggests that CUREs can benefit student skill development while assisting faculty by informing independent research projects and promoting publicity and awareness that may also benefit student recruitment and enrollment in additional courses and research projects.
The Genomics Education Partnership: Genuine undergraduate research experiences for online courses

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Since 2006, faculty members participating in the Genomics Education Partnership (GEP) have incorporated authentic genomics research experiences into the undergraduate curriculum, introducing thousands of students to eukaryotic gene structure, comparative genomics, and the evolution of Drosophila. The GEP is a consortium of over 150 diverse colleges and universities that provides Course-based Undergraduate Research Experiences (CUREs) for thousands of students from a variety of backgrounds. The GEP provides a centralized support system for students and faculty to engage in research in bioinformatics and genomics. The COVID-19 pandemic has forced many colleges and universities to provide remote instruction, presenting a challenge for faculty teaching biology laboratory courses. The GEP is uniquely positioned to address this challenge by providing research experiences in genomics for faculty and students within a completely online setting. Additionally, the GEP is providing remote, virtual teaching assistants for GEP students. Preliminary results from our faculty report suggest that the majority of GEP faculty teaching during the Spring 2020 semester find the GEP curriculum and infrastructure is well-suited for online instruction. The GEP assessment results from a pre/post quiz and survey administered in the Spring 2020 semester are similar to the results from previous semesters where most students were instructed face-to-face. However, even though only an internet connection and a computer are needed to complete a GEP research project, some students and faculty have limited access to reliable internet connections and computers. Moving forward, as we work to increase diversity in biology, inequities in access to technology will need to be addressed.

Teaching a cell and molecular biology laboratory on cloning during the COVID19 Pandemic

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There are many challenges to teaching a cell and molecular biology laboratory during a pandemic when students and faculty need to social distance and wear masks and face shields. They also cannot walk all around the room and congregate near the equipment in the laboratory. These restrictions make it difficult to hear and interact with the teacher and other students as well as making it difficult to perform many wet laboratories. The objective of this project is to show how to teach many of the same techniques and concepts during a pandemic with restrictions yet have the students learn many of the same concepts and skills. The students performed a few wet basic laboratories on campus to learn basic skills such as pipetting, electrophoresis, calculations and graphing. Afterwards, they performed a multiclass cloning project using the NEB website and other information posted by the professor. Many previous projects have shown that it is beneficial for the students to be involved with a multi class project. Previous experience has also shown that if students cannot complete one step of the project, it will impede moving on to the next step. By performing a multiclass experiment using websites, the students still learned about many steps in cloning but they were not limited by their technical ability that might have prevented them from moving to the next step in the project if they could not complete the prior step. The students were instructed to transfer a piece of DNA from the pUC322 plasmid to the
pUC19 plasmid. There was more than one way to perform this project. The students had the autonomy to design the experiment any way of their choosing as long as they followed certain rules that are part of any cloning project. They learned how the NEB website is a resource of information about restriction enzymes, plasmid maps, calculations and various protocols. Subsequent steps allowed the students to obtain hypothetical data about their project. By drawing gels, they could show if they ended up with just plasmid or with plasmid and insert. By the end of this project, the students learned the many steps of cloning and worked on problems even without actually performing many of the wet laboratories.

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Impacts of COVID-19 on mentored science research experiences in Summer 2020
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For students in the sciences, research experiences can be an important aspect of their education, and are linked to increased student awareness of career options, self-efficacy, and graduate school aspirations. With the onset of the Coronavirus Disease 2019 (COVID-19), the scientific community was forced to make modifications to academic and professional activities to accommodate “shelter in place” orders in 2020. This had a dramatic impact on planned science research experiences, forcing many mentors to transition their collaborations with students to a virtual format, while others canceled them altogether. We recruited 70 study participants engaged in research experiences at 19 U.S. institutions across 12 states during Summer 2020, conducting interviews and/or observations of virtual research meetings. In this study, the life sciences are most heavily represented. Interview data revealed that both mentors and mentees struggled with motivation and mental health issues, as well as resources (e.g., internet, housing, privacy, and childcare). Both mentors and mentees reported that the virtual environment made it challenging to build a rapport, as highly valued “spontaneous interactions” (e.g., dropping by someone’s office to check in) were missing. Mentees worried about the utility of a virtual research experience in helping them achieve their career goals, and reported feelings of “missing out” on networking with scientists and peers. Related to communication, mentees reported needing more support from mentors on their projects, though they felt uncomfortable asking questions outside of regularly scheduled meetings. Mentors reported feeling challenged with gauging their success as a mentor, training mentees remotely, and assessing mentee comprehension. There are some intriguing implications of virtual research experiences, such as cross-institutional collaborations, and increased access for students who do not wish to relocate. Preparation and/or training for mentors and mentees should address issues related to mental health and well-being, communication strategies, organizational tools, and ways for mentees to access technical resources and collaborators to support their work on the project. Mentors can encourage mentees to discuss their personal goals and motivations for participating in research, to support a strong mentor-mentee relationship and to increase the likelihood that the experience will be impactful for the mentee. We hope that improving virtual research experiences will help to reduce the negative impacts of COVID-19 on the future scientific workforce, and support productivity and well-being of both mentors and mentees.
Flexible utilization of remote lab kits in a hybrid teaching modality
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In response to the COVID-19 restrictions, science educators have faced challenges to design laboratory courses that deliver similar interactive hands-on learning experiences normally achieved with a face-to-face laboratory format. Many universities across the United States chose to deliver their curriculum fully online, and science education vendors (i.e. Wards, Carolina Biological) expanded their distance learning laboratory kit offerings to meet the needs for remote delivery. Barry University, a private Catholic university in Miami Florida, met the pandemic challenge by designing a "Flex-Hybrid" model for delivery of all classes—including labs. We developed a "hybrid" laboratory classroom, where both students and faculty had the freedom to attend the laboratory in-person or to attend remote, synchronous sessions. Thus, a single course could have two cohorts of student attendees (remote and in-person). Both student cohorts received distance learning lab supply kits that correspond to established learning outcomes for the lab courses. Students perform the experiments either remotely (synchronously) or in the laboratory with an in person teaching assistant and with the professor who may be teaching remotely or in person. A benefit of the “Flex Hybrid” model is the ability to pivot to fully remote instruction if an in-person student (or instructor) must temporarily attend remotely to quarantine. To examine student and faculty attitudes about the use of the distance learning laboratory kits in the Flex-Hybrid setting, we designed and developed two survey instruments that will be administered to instructors and students in the following lab courses: General Biology I and II, Foundations of Human Physiology, Microbiology for Health Sciences, Microbiology, Genetics and a Neurobiology for Non-Majors. The survey will be deployed mid-semester and at the end of the semester to students and faculty instructors. Preliminary feedback from students indicates that overall, students view the remote kits favorably and that they feel "a greater sense of responsibility" with the flex-mode of delivery for lab since they are working more independently than in a fully face-to-face laboratory setting. Faculty report that they have had opportunities to explore inquiry-based discussions on why participants may obtain different results with the same kit in different settings--something they do not get to discuss in a traditional face-to-face lab.

While the new “Flex-Hybrid” teaching mode was a necessary response to the current pandemic, the results of this study will help inform pedagogical approaches that ensure increased student learning in the context of our Biology labs.

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Virtual Reality for Cellular Neuroscience Medical Education
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Concepts of scale and spatial arrangement of cellular components are critical for medical student’s understanding of disease and effects of chemicals on the human body. Two dimensional illustrations and microscope images are generally the main method for conveying morphology at the cell and molecular level. Virtual reality and immersive three-dimensional visualizations are increasingly popular mechanisms for conveying microscopic tissue-cellular-molecular concepts in medical education. We report a new application of these uses of digital imaging and virtual reality with the important addition
of interactivity and self-customized learning. A group of medical students who had already completed neuroanatomy and neurobiology learning modules worked to build digital models of the morphology of the brain from macroanatomy to subcellular details of select brain regions using virtual reality-based sculpting. The digital anatomical sculptures they created allow examination of molecular cellular and tissue morphology across scales not possible in the laboratory or with static real-world models. The digital nature of the models the medical students built were then used for supplemental neuroanatomy training for the first year medical students during their 2020 neuroanatomy training. Students were able to record personalized video walk-throughs of the structures for subsequent study. We describe the workflow to allow learners to interact with and self-customize learning with smooth transition from macroanatomy to super-resolution microscope data to molecular level illustrations.

**Signaling in the Nervous System**

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**Activated CRHR1 triggers Akt phosphorylation in hippocampal neuronal cells**

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Corticotrophin releasing hormone (CRH) plays a key role in the regulation of neuroendocrine, autonomic, and behavioral response to stress. Dysregulation of the CRH/CRHR1 system is crucial in the pathogenesis of stress-related disorders. CRHR1 is a class B GPCR that signals mainly by Gs coupling, leading to an increase of cyclic AMP (cAMP) and the activation of multiple signaling cascades. Up to date the MAPKs pathway has been the most studied downstream of this receptor. We have previously described that CRHR1 activates G protein-dependent and receptor endocytosis-dependent mechanisms evidenced as a biphasic ERK1/2 activation in the mouse hippocampal cell line HT22 stably expressing CRHR1 (HT22-CRHR1). We have shown that upon stimulation, CRHR1 engages separate cAMP sources, involving the soluble adenylyl cyclase (sAC) in addition to transmembrane adenylyl cyclases. Both sources participate in the acute activation of ERK1/2, but only sAC activity is essential for the sustained phospho-ERK1/2 (pERK1/2) phase. Moreover, we demonstrated that CRHR1 continues to generate cAMP from endocytic compartments, uncovering a link between sAC and endosome based GPCR signaling. In the brain, Akt activity mediates several cellular processes, such as neuronal survival and synaptic plasticity. PI3K-Akt signaling has been implicated in the etiology of mood disorders as well as in the antidepressant-like effects of various psychiatric drugs. In this work we wanted to assess whether the PI3K-Akt pathway was involved in CRH/CRHR1 signaling and if there was a crosstalk with the MAPKs cascade. In HT22-CRHR1 cells, Akt was phosphorylated downstream activated CRHR1 in a cAMP-dependent manner. Phosphorylated Akt (pAkt) was active as evidenced by the phosphorylation state of substrates. pAkt profile was opposite to that of pERK1/2 which suggested a possible crosstalk between these pathways. Inhibition of MEK had no effect on Akt activation, whilst pharmacological inhibition of either PI3K or Akt diminished pERK1/2 levels in response to CRH. On the contrary, PI3K-Akt inhibition appeared to increase CRH-mediated cAMP response and CREB phosphorylation. Given that Akt activation temporally overlapped with the endocytosis-dependent phase of ERK1/2 activation, we evaluated pAkt levels in cells where internalization had been blocked and found that CRHR1 endocytosis is indeed required for Akt phosphorylation. These results indicate that PI3K-Akt pathway is activated
downstream CRHR1 in a neuronal context and that there is a crosstalk between Akt and ERK1/2. Moreover, Akt phosphorylation dependent on receptor internalization reinforces the idea of CRHR1 signaling from endosomes. Future work will involve the characterization of the endocytic compartments from which the receptor is signaling.

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Subcellular location-dependent activation and signaling of the delta-opioid receptor
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G protein-coupled receptors (GPCRs) transduce a wide array of cellular signals and have classically been thought to signal mainly from the plasma membrane. GPCRs also localize to multiple intracellular compartments, including endosomes and the Golgi. GPCR signaling from these intracellular compartments is an emerging idea in the field. The mechanisms by which GPCR localization to different cellular membranes promotes distinct signaling responses are not fully understood. We use the delta-opioid receptor (DOR) as a physiologically relevant example to address this question. DOR localizes to intracellular compartments, including the Golgi, in neuronal cells. We show, using live imaging, that fluorescently tagged conformational biosensors which read out active DOR conformations are differentially recruited to DOR in the plasma membrane and the Golgi. Further, plasma membrane and Golgi-localized DOR differ in the ability to regulate second messengers downstream of the receptor. Together, our data support a model in which DOR localization to the Golgi affects ligand-induced conformational changes in the receptor, as well as the activation of downstream signaling pathways. This spatial selectivity of the consequences of DOR activation may underlie the complexity of physiological effects associated with DOR activation and provide new ways to specifically modulate aspects of receptor signaling.

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The Prostaglandin E2 receptors EP2 and EP4 exhibit different signaling modalities leading to immunosuppressive or immunostimulatory dendritic cells.
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The incidence of melanoma has significantly increased over the past decade. Immunotherapy potentially restores patients’ own immune response against melanoma, but long-lasting responses occur in only ~30% of melanoma patients and the majority of patients exhibit weak/no response. This is mostly due to the presence of an immunosuppressive tumour microenvironment (TME), which dampens immunity and promotes tumour growth and dissemination. Despite the presence of dendritic cells (DCs) in melanoma lesions and their potential to generate anti-tumour immunity, TME-resident DCs often exhibit impaired or defective function. High Prostaglandin E2 (PGE2) levels in the TME might play a role since PGE2 promotes the production of immunosuppressive factors such as IL-10 in DCs. Yet, PGE2 induces the
highly migratory phenotype of mature DCs, which is crucial in immunity. Understanding PGE2 dual function in DCs can offer novel leads to reverse unwanted DC immunosuppression in the context of anti-tumour immunity. In DCs, PGE2 exclusively signals via EP2 and EP4 but the receptor-specific contributions are poorly defined. By using a FRET-based cAMP biosensor in cells exposed to different PGE2 concentrations before and after blocking EP2 or EP4 with specific antagonists, we discovered that EP2 and EP4 signaling modalities differ. Whereas EP4 induces a fast, high but transient cAMP production that is proportional to all PGE2 concentrations tested, EP2 induces a sustained cAMP production at PGE2 concentrations ≥1 μM. Moreover, EP2 and EP4 reciprocally control their signaling efficiency, and an intact microtubule network is required for cAMP production. Next we found that individual triggering of EP2 and EP4 leads to different immune outcomes in human DCs: EP4 is crucial for DC activation and maturation, whereas EP2 triggering leads to higher IL-10 and a more immunosuppressive phenotype. Finally, DCs exposed to melanoma conditioned medium upregulated EP2 but not EP4 expression. Our results strongly indicate that in DCs EP4 is the PGE2 concentration sensor, while EP2 responds only above a certain concentration, which could be a relevant difference within the melanoma TME. Our data further suggest that EP2 blockade could counteract PGE2-induced immunosuppression in DCs within the melanoma TME, while maintaining ‘beneficial’ EP4-mediated responses of DCs to PGE2.

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Viral Inhibitory Peptide of TLR-4 (VIPER) upregulates zymosan phagocytosis by microglia while promoting an anti-inflammatory response

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Microglia, the professional phagocytes in the central nervous system (CNS), play a critical role in maintaining homeostasis and their malfunction has been associated with various neurodegenerative diseases of the CNS, including Alzheimer’s Disease (AD). Hyperactive microglia with impaired phagocytic activity, alongside with the accumulation of amyloid-beta aggregates, are the hallmarks of AD. Emerging studies have proposed a fungal infection etiology to AD, associated with the presence of significant accumulations of Candida albicans (C.albicans) in brains of AD patients, suggesting impairment of fungal clearance by microglia as a possible cause of fungal accumulation. The main pathogen-associated molecular patterns (PAMPs) for C. albicans are the cell wall components, β-1,3 glucan, and O-mannan, which are recognized by pattern recognition receptors (PRRs), including β-glucan receptors such as Dectin-1, found on the surface of microglia. In this study, we showed that the pro-inflammatory PAMP Lipopolysaccharide (LPS), caused an upregulation in zymosan phagocytosis, a fungal cell wall extract from Saccharomyces cerevisiae, in the mouse derived BV2 microglia cell line. This upregulation was partially abrogated by blocking the β-glucan receptor with laminarin, a soluble β-glucan. To investigate the possible involvement of TLR-4, known to recognize LPS, in the upregulation zymosan phagocytosis, we treated BV2 cells with VIPER, known to specifically inhibit TLR-4 by binding to MyD88 Adaptor-Like and TRIF-Related Adaptor Molecule. Surprisingly, pre-treatment with VIPER not only failed to abrogate the LPS-induced upregulation of phagocytosis, but it significantly increased zymosan phagocytosis when added to BV2 cells on its own, while downregulating the production of pro-inflammatory markers NO and TNFα. The effect of VIPER was diminished when laminarin was added 15 min before adding zymosan, suggesting the upregulation of zymosan phagocytosis by VIPER is partially mediated by an increase in beta-glucan receptor activity. On the other hand, blockage of the extracellular domain of TLR-4 using anti-TLR-4 polyclonal antibodies, significantly diminished the effect of LPS on zymosan
phagocytosis, indicating the upregulation of phagocytosis by VIPER is probably mediated through an alternative signal transduction pathway. VIPER has been previously established as an effective anti-inflammatory molecule in rat models; our findings have possible applications in the development of therapies for AD, as they provide new information on the effects of VIPER on microglial function.

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**Regulation of lipid trafficking at endoplasmic reticulum-plasma membrane contact sites in photoreceptor neurons**

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At the distal regions of neurons, vesicular trafficking is inefficient to maintain lipid homeostasis and instead, the cell relies on contacts formed between closely apposed organellar membranes for lipid transfer. Perturbations of such contacts formed between the endoplasmic reticulum [ER] and mitochondria have been implicated in the progression of many neurodegenerative disorders. However, the mechanisms by which these contact sites directly contribute to maintaining neuronal physiology remain unclear. *Drosophila* photoreceptors are morphologically polarized neurons possessing contacts formed between the apical plasma membrane [PM] and ER that are indispensable for maintaining structural and functional integrity of the cell. RDGB is a key protein that tightly controls this process by transferring phospholipids and thus maintaining organellar lipid homeostasis. Here, we provide insight into how regulation of RDGB function directly impacts the physiology of photoreceptors. Electrophysiological recordings from photoreceptors devoid of RDGB function show significant reduction in light response and sensitivity, subsequently leading to their degeneration. We demonstrate that the function of RDGB at the ER-PM junction depends on multiple interactions. First, an intrinsically disordered region of RDGB supports its interaction with an integral ER protein, VAP. Second, the LNS2 domain of RDGB is essential to maintain its interactions with the apical PM. These interactions stabilize the protein at the ER-PM junction, while additional inter-domain movements within RDGB directly regulate its lipid transfer activity. Disruption of these interactions severely impacts lipid homeostasis leading to loss of structural and functional integrity of the photoreceptors. Our data thus provides insight into how regulation of lipid transfer is fine-tuned to maintain neuronal structure and function.

**Systems Cell Biology**

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**Machine learning based analyses of scRNAseq data characterize dynamical stability of cell phenotypes**

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Mammalian cells that share the same genome exist in notably distinct phenotypes, and the human cell atlas project aims to characterize all human cell types. Single cell transcriptomic data reveals a continuum of cell states, so a fundamental question is how to define a stable cell phenotype. Here we aim to address the question from the perspective of dynamical systems theories. Recent exciting developments in the single cell genomics enable measurements of transcriptome (x) and estimation of RNA velocity (instant time derivatives of transcriptome, dx/dt) of hundred thousands of cells. Through formulating as a machine-learning based repression problem, we developed a procedure to reconstruct
coarse-grained governing equations for stable cell phenotypes. Eigen analyses of the equations reveal collective dynamics of genes and coupling between different cellular processes. We are in the process of performing comparative studies about the collective modes of different cell phenotypes using available scRNA-seq data. The work will provide insight on cellular dynamics through analyzing transcriptome data with dynamical systems theories.

P460

**Signaling adaptation mediates rapid escape from BRAF inhibition in single melanoma cells**

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A non-genetic, reversible, drug-tolerant state has been reported both in the clinic and preclinical models, yet little is known about the inception of drug tolerance, in particular the timing and plasticity involved in cancer cell adaptation. Here we use time-lapse imaging and EllipTrack, a new automated cell tracking pipeline optimized for long-term tracking of hard-to-track cancer cells with minimal errors, to follow thousands of individual melanoma cells over the first four days of treatment with BRAF inhibitor. We find that while the majority of cells enter a CDK2-low quiescence, a subpopulation of cells can escape from drug action within three days to re-enter cell cycle. This escapee subpopulation reverts to a drug-sensitive state upon drug withdrawal, and cell lineages derived via EllipTrack-based cell tracking contain both escapees and non-escapees. These data suggest that rapid drug-induced signaling rewiring events, rather than genetic mutations, promote the ability of cells to divide in the presence of drug. To identify regulators involved in escape from BRAF inhibition, we perform single-cell RNA sequencing and computationally reconstruct the transcriptomic landscape in escapees by aligning the inferred trajectory of single-cell transcriptomes to the cell lineages measured by time-lapse imaging. We find that escapees display an AXL high/MITF low invasive gene signature and up-regulate multiple stress response pathways that enable their ability to cycle in drug. We further demonstrate that escapees are prone to mutagenesis yet outgrow non-escapees, suggesting that they could be the seed population driving the acquisition of permanent drug resistance. Together, this work provides real-time visualization of the extent of heterogeneity and plasticity in cancer-cell drug responses, and uncovers rewiring events that enable proliferation in drug, a key step on the road to drug resistance.

P461

**Nutrient signaling, stress response, and interorganelle communication are noncanonical determinants of cell fate**

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Isogenic cells can manifest distinct cellular fates for a single stress, however the nongenetic mechanisms driving such fates remain poorly understood. Here we utilize the response of individual budding yeast cells to acute glucose removal (AGR) to unveil the nongenetic determinants in cellular heterogeneity. We use a time-lapse imaging platform coupled to a microfluidics device and a computational analysis pipeline that allows us to continuously track single cells before, during, and after AGR for 16 hrs. We show that individual cells respond differentially to AGR: upon glucose replenishment, some cells proliferate and resume budding (quiescent cells) while others do not resume budding despite being metabolically active (senescent cells). We simultaneously tracked nutrient signaling, cell cycle, stress,
metabolic, and interorganelle contact site markers, dissecting key cellular determinants for response to AGR. We show that all cells halt or delay their cell cycle upon AGR regardless of the cell cycle stage they are in. Senescent cells activate stress responses upon AGR, but achieve a distinct cellular response compared to quiescent cells. In particular, senescent cells display altered interorganelle crosstalk as evidenced by their inability to expand their nucleus-vacuole junctions (NVJ) and exhibited defects in endomembrane trafficking. However, senescent cells remain responsive to environmental cues throughout and after AGR stress, yet fail to proliferate upon glucose replenishment. In line with this, both senescent and quiescent cells respond to nutrient deprivation by up-regulating lipid storage pathways in the form of lipid droplets (LDs), although we surprisingly find that LDs are not operationally required for post-AGR cell fates. Remarkably, we demonstrate that the abundance of the nutrient signaling related kinase Rim15 before AGR predicts cell fate after AGR. Finally, we accurately predicted the fates of individual cells during AGR hours before their cell fates manifest, by applying the Bayesian method of statistical evidence to our single-cell measurements. We also show that cells ‘decide’ on their fates within 3-4 hours of the initiation of the AGR. Collectively, our work pioneers a shift in focus from cell fate-specific signaling to other noncanonical factors related to nutrient signaling, interorganelle communication, and stress response and shows that cellular responses are predisposed to particular fates well before the fates manifest.

P462

A plausible accelerating function of intermediate states in cancer metastasis

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Epithelial-to-mesenchymal transition (EMT) is a fundamental cellular process and plays an essential role in development, tissue regeneration, and cancer metastasis. Interestingly, EMT is not a binary process but instead proceeds with multiple partial intermediate states. However, the functions of these intermediate states are not fully understood. Here, we focus on a general question about how the number of partial EMT states affects cell transformation. First, by fitting a hidden Markov model of EMT with experimental data, we propose a statistical mechanism for EMT in which many unobservable microstates may exist within one of the observable macrostates. Furthermore, we find that increasing the number of intermediate states can accelerate the EMT process and that adding parallel paths or transition layers may accelerate the process even further. Last, a stabilized intermediate state traps cells in one partial EMT state. This work advances our understanding of the dynamics and functions of EMT plasticity during cancer metastasis.

P463

Heterogeneous CXCL10 expression in macrophages in response to IFNγ stimulus

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Macrophages are innate immune cells that coordinate responses to inflammatory stimulus, synthesizing signals from their environment to induce gene expression cascades leading to diverse functions. Interferon-gamma (IFNγ) is a pro-inflammatory cytokine that macrophages encounter during both the innate and adaptive immune responses and induces macrophages to secrete further cytokines and...
chemokines. One of these chemokines, CXCL10, is important for recruiting T cells to the site of inflammation. This study quantitatively investigates how macrophages decode different concentrations and pulse lengths of IFNγ stimulus into downstream CXCL10 expression. To study this at a single-cell level, I image live macrophages containing endogenous fluorescent reporters for CXCL10 and its upstream transcription factor IRF1 in a microfluidic device. I find that IRF1 and CXCL10 respond differently to lower doses of IFNγ, with lower IFNγ doses leading to a greater delay in CXCL10 expression than in IRF1 expression. Further, I find heterogeneity in expression of CXCL10, with some macrophages expressing CXCL10 in response to IFNγ while others do not, and that lower IFNγ doses lead to a smaller percentage of macrophages expressing CXCL10. However, IRF1 expression is not heterogeneous. I conclude that there are factors other than IRF1 leading to heterogeneity in CXCL10 expression and that macrophage populations tune this heterogeneity in CXCL10 expression to respond appropriately to the IFNγ stimulus.

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Comparing Transcriptome Profiles of Cadmium Selenide/Zinc Sulfide and Indium Phosphide/Zinc Sulfide in Saccharomyces cerevisiae

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The primary focus of our research is to obtain global gene expression data in baker’s yeast exposed to sub-lethal doses of specific quantum dots (QDs), such as green emitting CdSe/ZnS and InP/ZnS, to reveal novel insights on their mysterious and controversial mechanisms of toxicity. Despite their promising and ever-growing list of applications (fluorescent probes, drug delivery systems, etc.), their potential toxicity and long-lasting effects on the environment are not well understood. Due to the versatile nature of nanoscale materials, their mechanisms of toxicity can differ vastly based on their physical characteristics. To assess toxicity, we conducted cell viability assays, ROS detection assays, apoptosis experiments, and assessed the effects of each QD on trans-Golgi and transmembrane vesicle sorting pathways with GFP-tagged proteins and confocal microscopy. Most importantly, we used high-throughput technology, such as RNA-sequencing, to obtain gene expression profiles and the identities of specific differentially expressed genes (DEGs) in yeast exposed to each QD, respectfully. We found CdSe/ZnS QDs significantly altered genes implicated in carboxylic acid, amino acid, nitrogen compound, and protein metabolic processes, along with processes such as transmembrane transport, cellular homeostasis, cell wall organization, translation, and ribosomal biogenesis. Additionally, we found InP/ZnS QDs to alter genes associated in oxidation-reduction, transmembrane transport, metal ion homeostasis, cellular component organization, and translation processes along with protein and nitrogen compound metabolic processes. Interestingly, we observed no change in ROS production in CdSe/ZnS treated cells and a significant decrease in cellular superoxide production and significant increase in cellular peroxynitrite production in InP/ZnS treated cells. RT-qPCR was also conducted to validate RNA-seq data by assessing the expression of significantly up- and down-regulated genes along with an unaffected housekeeping gene. In summation, we found InP/ZnS QDs to be more toxic than CdSe/ZnS QDs on Saccharomyces cerevisiae.
Mapping vector field of single cells
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Gene regulatory networks (GRN), consisting of interlocking feedback loops, govern cellular dynamics and cell fate decisions. A paramount task in systems biology is to learn the governing equations defined by the network structure. Decades of research on GRN has demonstrated that this is an unfathomable challenge. Recent exciting developments in the single cell genomics enable measurements of transcriptome (x) and estimation of RNA velocity (instant time derivatives of transcriptome, dx/dt) of hundred thousands of cells at unprecedented spatiotemporal resolution, and open the door to sufficiently powered inference. Building upon a comprehensive framework, dynamo (https://github.com/aristoteleo/dynamo-release), we fully takes advantage of the novel metabolic labeling based single-cell RNA-seq (scSLAM-seq) to improve the estimation of RNA velocity. With dynamo, we also show that genome-wide vector field function can be accurately and efficiently reconstructed with the improved velocity estimations, which is then used to predict cell fate over an extended time period. We applied dynamo to several conventional scRNA-seq data sets that span hematopoiesis, zebrafish pigmentation, intestinal organoid differentiation, etc, our own scSLAM-seq data on human HL60 differentiation and all other public metabolic labeling based scRNA-seq to map the vector field functions in those studies. We further extracted topological features of vector fields of these systems such as fixed points and separatrices. Analyzing the corresponding functional acceleration field, divergence, and Jacobian matrix of interacting genes in each cell revealed key regulation relations across those systems. Our memory-seq studies further confirm the reconstructed vector field by reliably predicting cell state evolution over a long time, as measured by the sister or cousin cells sampled from different time points. Our method of single cell vector field reconstruction thus contributes as a significant step towards the holy grail of learning the governing equations of any cellular dynamic processes.

The Inner Nuclear Membrane

Biological Aging Dependent Differential Nuclear Dynamics
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Cells sense and respond to extracellular mechanical cues to govern the mechano-homeostasis that is important in regulating various cell behaviors. Aging is a multifaceted degenerating process involving numerous functional and structural changes in a cell over time. Aging induced disrupted mechano-homeostasis ultimately results in the progression of chronic diseases such as cardiovascular, neurodegenerative disorder. Altered mechanical properties of aging cells cause physiological dysfunctions such as attenuated cellular ability to recognize their micro-environment and to recruit
physical stimuli into biochemical signaling. While previous studies of aging largely relied on clinical aspects, cellular and subcellular structure and functions based on mechanobiological perspectives remains missing. Here we first identify the molecular basis of attenuated cell mechanosensation. For systematic analysis of cell morphology, automated high-throughput cell phenotyping technique is applied, revealing that the differential characteristics of nuclear dynamics depend on biological aging. Moreover, chromatin dynamics proves that the cell’s ability to recognize their surroundings is closely associated with aging process. Thus, expression of aging-associated nuclear proteins and nuclear morphology differently change with the donor’s age in response to external physical cues. Our results suggest that age-related deterioration of the cellular-response machinery is regulated by intracellular molecular connections. We expect that the understanding of the connections between these age-related cellular changes and mechanical cues is expected to determine the donor’s biological age, which is critical to suggest new pathways to target in the development of novel prevention and treatment strategies.

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Effects of prelamin A on fibroblast polarization
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Lamins structurally support the inner nuclear membrane and influence various nuclear, intracellular and extracellular processes. Hutchinson-Gilford progeria syndrome (HGPS), a premature aging syndrome, is caused by mutation in LMNA encoding lamin A and lamin C. Lamin A is synthesized as a precursor, prelamin A, whose carboxyl-terminus undergoes farnesylation and other modifications before being cleaved off by zinc metallopeptidase STE24 homolog (ZMPSTE24). A prelamin A variant (termed progerin) lacking a 50-amino acid fragment containing the cleavage site is expressed in cells of patients with HGPS. Therefore, farnesylated progerin accumulates at the inner nuclear membrane. Farnesylated prelamin A (with a single amino acid change) also accumulates in cells from a patient with a progeroid disorder (PD) carrying an LMNA point mutation disrupting the ZMPSTE24 cleavage site. Likewise, mutations in ZMPSTE24 leading to partial loss of proteolytic activity result in prelamin A accumulation in mandibuloacral dysplasia type B (MADB). The PD and MADB patients have phenotypes overlapping with but less severe than HGPS. Hence, the accumulation of prelamin A may not lead to exactly the same cellular defects observed in HGPS. Previously we showed that nesprin-2 and SUN2-dependent nuclear movement and centrosome orientation (“cell polarity”) in migratory fibroblasts were inhibited by progerin due to elevated association between the nucleus and microtubules (Chang, W. et al., PNAS, 2019). Here we find that the diffusional mobilities of nesprin-2G and SUN2 are affected by the accumulation of prelamin A in fibroblasts from the PD and MADB patients, but only fibroblasts from the MADB patient exhibit a significant cell polarity defect. Cell polarity is also impaired in embryonic fibroblasts from Zmpst24-null mice and NIH3T3 cells depleted of ZMPSTE24 using siRNAs. Cell polarity defects in these cells could be rescued by inhibiting protein farnesylation. As in HGPS fibroblasts, these defects can also be rescued by inhibiting dynein. These result show that farnesylated prelamin A and progerin similarly affect fibroblast polarization.
Structure-function analysis of potential lamin packing interfaces in \textit{C. elegans}

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Lamins form the supportive skeleton that gives the nucleus its shape, provides mechanical protection against deformation in response to force, and ensures proper gene expression. Point mutations within lamin genes are known to cause muscular dystrophies, but the mechanism by which these mutations cause disease is unclear. Using the single \textit{C. elegans} lamin, the Schwartz lab crystallized 300aa of its 350aa coiled-coil and found that neighboring coiled-coil dimers form packing interfaces. These interfaces harbor a significant amount of conserved amino acids which are mutated in Emery-Dreifuss muscular dystrophy (EDMD). We hypothesize that mutations which disrupt packing interfaces will interfere with lamin superstructure assembly, phenocopying EDMD. Using CRISPR-Cas9, we individually mutated seven conserved amino acids within these packing interfaces to alanines and assayed motility defects by observing swimming in L4 and 9 day old worms. One mutation disrupted viability, while another significantly impacted motility. However, most point mutations exhibited no obvious phenotypes in viability or swimming assays. We are currently creating higher-order mutants predicted to completely abolish tertiary structure at these packing interfaces to determine their physiological relevance.

Lamin B1 Dynamics are Differentially Sensitive to Levels of Specific Nuclear Envelope Proteins In iPSC and Germ Layer Cells

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The nuclear lamina is a dynamic, fibrous structure found on the innermost side of the inner nuclear membrane in animal cells where it contributes to proper nuclear function. Mutations that affect lamina structure are known to cause a number of genetic diseases termed laminopathies, including Hutchinson-Gilford Progeria, muscular dystrophies, lipodystrophies, and neuropathies. It is largely unknown how these laminopathies can affect such a wide range of different tissues, and questions remain about how the structure and function of the lamina differ depending on cell type. As cells differentiate, the lamina generally incorporates more lamin A/C and less lamin B1, and it is known that the specific DNA regions that interact with the lamina vary between cell types. Because less is known about nuclear lamina dynamics, we examined cell type-dependent changes in the dynamics of an important nuclear lamina protein: lamin B1. Specifically, we measured GFP-lamin B1 dynamics using FRAP, finding that lamin B1 dynamics differ in iPSC and germ layer cells. Furthermore, we observed that lamin B1 dynamics in iPSC and germ layer cells respond differently to reduced expression of specific nuclear envelope proteins, namely lamin B receptor (LBR), emerin, ELYS, and lamin B2. For instance, simultaneous knock-down of LBR and ELYS increased the total fluorescence recovery of GFP-lamin B1 at the nuclear envelope by 83% in iPSCs, while no significant change was detected in mesoderm cells. This information, combined with our previous data examining differential changes in lamin B1 dynamics in response to PKC activation in these cell types, suggests that different nuclear envelope proteins determine lamin B1 dynamics depending on the differentiation state. Our data support the notion of cell type-dependent differences in nuclear
lamina structure and dynamics, differences that may underlie the diverse range of phenotypes observed in laminopathies.

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**Meiotic remodeling of the inner nuclear membrane proteome**

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The inner nuclear membrane (INM) proteome regulates nuclear function through its roles in gene expression, chromatin organization, and nuclear transport. Changes in the protein composition of the INM relative to developmentally regulated processes such as gametogenesis are poorly defined. Here, we examined all transmembrane proteins in *Saccharomyces cerevisiae* using a split-GFP complementation system to define the INM protein composition in gametes. Our results show that the INM proteome in gametes is distinct compared to the INM proteome in mitotic cells. Our diploid strain that was induced to undergo meiosis contained one tagged copy of the gene and one WT copy of the gene. The haploid meiotic products, of all positive proteins examined, expressed either a tagged copy of the gene or a WT copy of the gene, supporting *de novo* INM protein synthesis in gametes as opposed to inheritance of proteins from the mother cell. We found that most proteins present in gametes but absent in mitotic cells are required for complete gamete formation. In addition, whereas mitotic cells show a strong preference for small nuclear domains of single pass transmembrane proteins, gametes do not have a preference for small nuclear domains, contrary to the current paradigms of nuclear transport. Our data further demonstrate that there is a loss in the nuclear permeability barrier during gametogenesis that is coupled to *de novo* nuclear pore complex (NPC) biogenesis. Together, our findings define gametogenesis-specific nuclear remodeling of the INM proteome where necessary proteins for gamete formation are newly expressed.

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**The ESCRT machinery directs quality control over inner nuclear membrane architecture**

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The nuclear envelope (NE) exhibits a unique membrane topology, consisting of two distinct lipid bilayers that are joined at nuclear pore complexes, which regulate nucleocytoplasmic transport during interphase. Prior to mitotic spindle assembly, the NE disassembles in metazoans and must subsequently reform around segregated masses of chromatin in a process that requires a series of dramatic membrane remodeling events. The late-acting endosomal sorting complex required for transport (ESCRT) machinery has been implicated in facilitating the resealing of the NE after mitosis, enabling compartmentalization of the genome away from the cytoplasm. Here, we leverage the first stereotypic division of the *C. elegans* one-cell stage embryo to identify additional functions of the ESCRT machinery in maintaining the structure of the inner nuclear membrane. Specifically, impaired ESCRT function results in a defect in the pruning of inner nuclear membrane invaginations, which form normally after NE reformation. Additionally, in combination with a hypomorphic mutation that interferes with
assembly of the underlying nuclear lamina, inhibition of ESCRT function significantly increases chromosome segregation defects, resulting in penetrant embryonic lethality. Our findings provide a new link between ESCRT-mediated inner nuclear membrane remodeling and maintenance of genome organization and integrity.

Vesicle Docking and Fusion

P472

Ubiquitination of SNARE proteins mechanically regulates neurotransmitter release by increasing entropic forces that drive membrane fusion

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Here we present evidence that ubiquitin increases the neuronal vesicle release probability by increasing entropic forces among SNARE proteins that drive vesicle-plasma membrane fusion. Ubiquitination adds bulky attachment groups to SNAP-25, increasing its effective size and thereby increasing the entropic forces. NT release at a neuronal synapse is evoked by an action potential that opens voltage-gated Ca-channels to raise the intracellular Ca concentration. Raised [Ca] then triggers a specialized machinery to fuse vesicles with the plasma membrane, releasing their contents into the synaptic cleft. SNARE proteins are the core of this membrane fusion machinery. Typically, one action potential evokes fusion of only ~5-10% of the release-ready vesicles in the active zone with the plasma membrane. Many factors that regulate this vesicle release probability, Pves, have been identified. These include the SNARE proteins themselves and the ubiquitin proteasome system. Here, we present experimental and theoretical results demonstrating that ubiquitination of the SNARE protein SNAP-25 increases the release probability Pves by enhancing entropic forces that drive membrane fusion. We took electrophysiological recordings from hippocampal autaptic cultures expressing ubiquitination-resistant mutants of SNAP-25, mutating putative ubiquitination sites to prevent their ubiquitination. Mutating ubiquitination sites at the C-terminal end of the SN1 helix of SNAP-25 significantly impaired NT release. In autaptic cultures expressing the SNAP25-K72R and SNAP25-K76R mutants, excitatory post-synaptic current (EPSC) amplitude and charge was significantly decreased. In cells expressing the SNAP25-K72R mutant, this was associated with a three-fold reduction in vesicle release probability, while in the SNAP25-K76R mutant, it was associated with a similar reduction of the readily-releasable pool. To rationalize these results, we performed molecular dynamics simulations of SNARE-mediated membrane fusion using highly coarse-grained representations of ubiquitin and the neuronal SNARE complex to access the long timescales of NT release. SNARE complexes rapidly self-assembled into a ring-like arrangement, with their C-termini at the center and N-termini at the outer edge. Entropic forces drove this ring to expand, pressing the membranes together and fusing them. Removal of ubiquitin from either the K72 or K76 residue at the C-terminal end of the SNARE complex weakened the entropic forces driving fusion by decreasing crowding at the center of the ring, thus significantly lowering the rate of SNARE-mediated fusion. Due to the lowered fusion rate, the total probability of vesicle release during the transient action potential was reduced, in agreement with experiment.
Ultra coarse-grained simulations support the entropic force hypothesis for SNARE-mediated membrane fusion

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Membrane fusion is an essential step for many basic cellular processes such as neurotransmitter or hormone release and trafficking. Cells achieve this using a multi-component fusion machinery whose core components are the SNARE proteins, often called the minimal fusion machinery. The mechanism of cooperative SNARE-mediated membrane fusion are not established, and the intermediate states along the fusion reaction pathway remain controversial. Computer simulations can help uncover the basic mechanisms, but a major obstacle is that the timescales of membrane fusion in physiological and in vitro settings range from msec to sec, far beyond the capabilities of all-atom or widely used coarse-grained (CG) approaches such as Martini. Here we used ultra-coarse-grained (UCG) simulation approaches to address the mechanisms of membrane fusion, using the Cooke-Deserno force field 4-bead UCG representation of phospholipids (Cooke et al., 2005). We incorporated a series of idealized fusogens into vesicles built from these UCG phospholipids, culminating in UCG SNARE complexes, which allowed us to examine fusion mechanisms up to msec timescales and beyond. We find that fusion is determined by a tension between two opposing tendencies. On the one hand, the SNARE complexes tended to zipper down to the point of closest approach between the two vesicles and hence separate the vesicles, an unproductive state. On the other hand, entropic forces among the SNARE complexes and membranes tended to push the SNAREs outwards; provided the SNARE linker domains were not too long, this brought the vesicles into such close contact as to promote their fusion via a hemifused stalk intermediate at the edge of the contact zone that expanded along the contact zone edge into a fusion pore. Thus, bulky SNARE complexes are necessary because they create entropic forces that clear out the contact zone between the two vesicles, preventing aggregation of the transmembrane domains at the point of closest approach between the two membranes. Fusion was promoted by increasing the number of UCG SNARE complexes, or by artificially increasing the diameter of the SNARE-like fusogens. These effects agree with previous studies suggesting that SNARE-mediated membrane fusion is driven by entropic forces created by crowding of the SNARE complexes at the fusion site (Mostafavi et al., 2017; McDargh et al., 2018).

Energetics and composition of nanoscale membrane pores mediate exocytosis, drug delivery and transfection

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Biological membranes owe their strength and low permeability to the phospholipid bilayers at their core. Membrane strength is determined by the energetics and dynamics of membrane pores, whose tension-dependent nucleation and growth leads to rupture. Creation of nanoscale membrane pores is central to exocytosis, trafficking and other processes fundamental to life that require breaching of secure plasma or organelle membranes, and is the basis for biotechnologies using drug delivery, delivery of genetic material for gene editing and antimicrobial peptides. A prevailing view from seminal
Electroporation and membrane rupture studies is that pore growth and bilayer rupture are controlled by macroscopically long-lived metastable defect states that precede fully developed pores. It was argued that defect nucleation becomes rate-limiting at high tensions, explaining the exponential tension-dependence of rupture times [E. Evans et al., *Biophys. J.* 85, 2342-2350 (2003)]. Here we measured membrane pore free energies and bilayer rupture using highly coarse-grained simulations that probe very long time scales. We find no evidence of metastable pore states. At lower tensions, small hydrophobic pores mature into large hydrophilic pores on the pathway to rupture, with classical tension dependence of rupture times. Above a critical tension membranes rupture directly from a small hydrophobic pore, and rupture times depend exponentially on tension. Thus, we recover the experimentally reported regimes, but the origin of the high tension exponential regime is unrelated to macroscopically long-lived pre-pore defects. It arises because hydrophilic pores cannot exist above a critical tension, leading to radically altered pore dynamics and rupture kinetics. We discuss the implications of our results for exocytosis, during which the pathway to membrane fusion may pass through a hemifusion diaphragm intermediate whose rupture leads to fusion via simple pore nucleation and growth. Our results also relate to drug delivery and transfection, which we find are more efficient at lower osmolarity conditions due to increased membrane tension that increases the number of hydrophobic pores whose opening is much easier than hydrophilic pores.

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**Neurotransmitter release: How many Synaptotagmin molecules does it take to clamp a SNARE complex?**

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Neurotransmitter (NT) release is achieved by a specialized protein machinery that senses presynaptic Ca-entry following an action potential, and responds by fusing vesicle and plasma membranes, releasing vesicle contents through the resultant fusion pore. Synaptotagmin 1 (Syt) is the Ca sensor for synchronous NT release, and is thought to act by inhibiting (“clamping”) the fusion action of the SNARE proteins at basal intracellular [Ca], then releasing the clamp at elevated [Ca]. (This is corroborated by the fact that SNARE proteins constitutively fuse membranes in the absence of inhibition.) While synaptic vesicles are known to host ~15-20 Syt molecules and ~70 copies of the SNARE protein VAMP, the number of assembled SNARE complexes involved in the fusion reaction is controversial, and the number of Syt molecules clamping the SNAREs is unknown. Here, we present coarse-grained molecular dynamics simulations of NT release, varying the number of assembled SNARE complexes and Syt molecules. In simulations of Ca-uncaging, the apparent cooperativity (Hill coefficient) of release was roughly proportional to the stoichiometric ratio of Syt molecules to SNARE complexes, with a ratio of 2:1 reproducing the well-known 4th power scaling of post-synaptic current amplitude with intracellular Ca-concentration. Varying the number of SNAREs and Syt molecules at a fixed ratio, we find that increasing the number of Syt-SNARE “modules” does not alter the cooperativity, but increases both the action-potential evoked release probability, Pves, and the Ca-sensitivity of NT release. Our results recapitulate experimental findings in which the number of SNARE complexes per vesicle was varied in the Calyx of Held, and in autaptic cultures (Acuna et al., 2014; Arancillo et al., 2013). Why did more SNARE complexes increase the vesicle release probability Pves? What is the mechanism? Prior to Ca-entry, Syt is assumed organized as a ring-like oligomer (Wang et al., 2014). Elevation of the presynaptic [Ca] catalyzed rapid Syt ring disassembly and unclamping of the SNARE complexes. The unclamped SNARE
complexes then spontaneously assembled into a ring. Entropic forces expanded the SNARE ring, pulling the membranes together and fusing them. These entropic forces were enhanced with more SNARE complexes, accelerating fusion. This last finding reveals the mechanism by which the release probability $P_{ves}$ increases with more SNARE complexes: during a transient action potential, the intracellular Ca concentration is only transiently elevated, and vesicles are only temporarily unclamped: there is a limited window of time during which fusion can occur. Thus, with faster fusion, the fraction of vesicles that fuse before the window is closed and the intracellular [Ca] returns to resting levels is increased.

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TRIM67 Regulates Exocytic Mode and Neuronal Morphogenesis via SNAP47

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Exocytosis has been of interest to cell biologists and developmental neuroscientists in awe of the plasma membrane expansion concomitant with neuronal morphogenesis. Whether different modes of fusion occur in developing neurons has never been asked. In contrast, two modes have been argued to occur at the synapse. During full vesicle fusion (FVF) exocytosis, the entirety of vesicle contents are secreted and the vesicle membrane incorporates in the plasma membrane. In contrast, during kiss and run fusion (KNR) a transient fusion pore secretes lumenal cargo, but closes without membrane addition. In the developing neuron we hypothesized that FVF events fuel membrane expansion, whereas KNR events do not. The controversy over the contribution and existence of two fusion modes has been maintained due to the nature of synaptic transmission, with rapid bursts of multiple fusion events clustered in the diffraction-limited synapse, and the lack of mechanistic insight into how mode might be dictated. Fortuitously, in developing neurons, exocytic events are spatially and temporally discrete making this analysis much more tractable. Here, we resolve individual exocytic events in developing murine cortical neurons prior to synaptogenesis, and use a newly developed, rigorous and unbiased classification tool to differentiate modes of fusion. To our surprise, we identified four modes. We demonstrate all modes comprise bona fide exocytic events, with two FVF-like classes and two KNR-like classes. Classes could be differentiated by their distinct fluorescence decay, suggestive of distinct behavior of the fusion pore. We quantify respective contributions to developmental exocytosis and develop simulations to model plasma membrane expansion. Consistent with our hypothesis, FVF-like exocytosis predominates when plasma membrane expansion is rapid. Both simulations and experimental evidence agree that reducing the contribution of FVF slows plasma membrane expansion and morphogenesis. Mechanistically we identify that the E3 ubiquitin ligase TRIM67 promotes FVF-like exocytosis by limiting incorporation of the Qb/Qc SNARE SNAP47 into SNARE complexes and thus, SNAP47 involvement in exocytosis. Using neurons from $^{Trim67^{-/-}}$ embryos, we observed increased contributions of KNR-like fusion and consequently reduced plasma membrane expansion. This was concomitant with increased SNAP47 protein levels and incorporation in exocytic SNARE complexes. We show that SNAP47 is necessary and sufficient to alter exocytic mode. Our data suggest that SNAP47-containing SNARE complexes temporarily arrest the fusion pore in an open state, resulting in increase in KNR events. These findings advance our understanding of different modes of fusion and their contribution to neuronal morphogenesis.
Proximity-induced membrane structure rearrangements precede membrane hemifusion


Biological membrane merger through fusion is essential in processes such as neurotransmission, fertilization, or viral infection. Diverse families of proteins catalyze membrane fusion through a common pathway that includes docking, merger of proximal leaflets (stalk formation and hemifusion), and formation of a fusion pore. Short lifetime of fusion intermediates makes them difficult to study and for that reason especially steps immediately before hemifusion remain largely unexplored. Here, we utilized in vitro reconstitution in combination with arresting point mutations in SNARE proteins in order to characterize the properties and structure of docked states preceding hemifusion. With the aid of interferometric scattering microscopy, we observed diffusional slowing down of vesicles arrested in consecutive fusion stages. Moreover, with light and cryo-electron microscopy combined with atomistic molecular dynamic simulations we were able to describe structural changes in membranes arrested during tight docking. We demonstrate formation of a stable, protein-free docking intermediate characterized by local membrane thickening. Taken together, these results present novel features of membrane-membrane interactions prior to formation of a fusion stalk that could potentially be utilized by cells to regulate membrane fusion speed and efficiency.

Mechanochemical sequestration maintains membrane homeostasis during exocytosis of large secretory vesicles

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Maintaining membrane homeostasis in secretory epithelial tissues, where continuous fusion of vesicles with the apical membrane takes place, can be challenging. This is most dramatically demonstrated in exocrine tissues that secrete various cargoes from large vesicles, ranging up to 10 microns in diameter. Secretion of such giant vesicles adds large amount of membrane to the apical surface of cells. Yet, it remains unclear how homeostasis of the cell surface in terms of size, shape and composition is maintained under these extreme circumstances. To address this question, we use the Drosophila larval salivary glands and the mouse acinar pancreas as complementary model systems for settings in which secretion via large vesicles takes place. Live-imaging and correlative light and electron microscopy (CLEM) revealed that after vesicle fusion to the apical surface, the vesicular membrane folds into a compact structure that retains the membrane while the content is released. Membrane folding and retention is mediated by an actomyosin coat, which is recruited to the vesicle after fusion. Fluorescence recovery after photobleaching (FRAP) experiments in the Drosophila salivary glands showed that there is restricted diffusion of transmembrane and lipid-anchored proteins between the fused vesicular and
apical membranes, suggesting that a diffusion barrier forms after fusion to insulate the vesicular membrane. Clathrin-mediated endocytosis (CME) is specifically recruited to the left-over, empty, and folded membrane to internalize the membrane and protein components. Taken together, these results demonstrate that both mechanical and chemical sequestration of the vesicular membrane maintain plasma membrane homeostasis during exocytosis of large secretory vesicles. Our findings also reveal that the membrane of large secretory vesicles does not integrate into the surface and that the role of actomyosin is to fold the membrane such that the content is extruded. Since we observed this novel mode of exocytosis in both Drosophila and mice tissues, we conclude that it is likely a conserved mechanism used by large exocrine vesicles across evolution.

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Mutations in the exocyst component EXOC2 cause severe defects in human brain development

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Vesicle transport is a fundamental biological process that enables proteins in membrane-bound vessels to move between cellular compartments, including the outer cell membrane. The exocyst is an eight-protein complex found in almost all plants and animals and is essential for tethering vesicles to the outermost membrane of cells so as to promote their delivery. Loss of function of any component of the complex is lethal in multiple organisms. Here we report pathogenic variants in an exocyst subunit, EXOC2 (Sec5)*. Affected individuals have severe developmental delay and brain abnormalities. Family 1 had two offspring with a homozygous truncating variant in EXOC2 that leads to nonsense-mediated decay of EXOC2 transcript, a severe reduction in exocytosis and vesicle fusion, and undetectable levels of EXOC2 protein. The patient from Family 2 had a milder clinical phenotype and reduced exocytosis. Cells from both patients showed defective Arl13b localization to the primary cilium. The discovery of mutations that partially disable exocyst function provides valuable insight into this essential protein complex in neural development. Since EXOC2 and other exocyst complex subunits are critical to neuronal function, our findings suggest that EXOC2 variants are the cause of the patients’ neurological disorders. * Van Bergen, N. J. and Ahmed, S.M. et al. Mutations in the exocyst component EXOC2 cause severe defects in human brain development. J. Exp. Med. (2020). doi:10.1084/jem.20192040

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Transmembrane peptidase CD13 is a negative regulator of cell-cell fusion in osteoclastogenesis

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Bone homeostasis is a complex process maintained by the balance of activity of two specialized cell types: the bone-generating osteoblasts and bone-degrading osteoclasts which must act in a tightly controlled and sequential manner to preserve skeletal health. Osteoblasts produce the soluble receptor activator of NFκB ligand (RANKL) that binds to its receptor RANK on the surface of osteoclast precursor cells to promote osteoclastogenesis, a critical process for skeletal growth and development that involves cell-cell fusion and assembly of molecular machinery that ultimately leads to resorption of bone. CD13 is
a transmembrane aminopeptidase that is highly expressed in cells of myeloid lineage. CD13 regulates
dynamin-dependent receptor endocytosis and recycling and actively contributes to actin cytoskeletal
organization. In the present study, we demonstrate that despite a normal distribution of osteoclast
progenitor populations in the bone marrow and periphery, CD13-deficient mice display a low bone
density phenotype with increased osteoclast numbers per bone surface area, which was significantly
augmented in aged mice lacking CD13. Further, the endosteal bone formation rate is similar between
genotypes, indicating a defect in osteoclast-specific function in vivo. Loss of CD13 led to exaggerated in
vitro osteoclastogenesis as indicated by significantly enhanced fusion of bone marrow-derived
multinucleated osteoclasts in the presence of M-CSF and RANKL, resulting in abnormally large cells
containing remarkably high numbers of nuclei with a concomitant increase in bone resorption activity.
Mechanistically, while expression levels of the fusion-regulatory proteins dynamin and Dendritic Cell-
Specific Transmembrane Protein (DC-STAMP) are normally downregulated as fusion progresses in
fusion-competent mononucleated bone marrow progenitor cells, these are aberrantly sustained at high
levels in the absence of CD13, even in mature multi-nucleated osteoclasts. Taken together, we conclude
that CD13 negatively regulates cell-cell fusion during osteoclastogenesis by controlling expression and
localization of key fusion-promoting proteins that are critical for osteoclast fusion. We propose that
CD13 may serve as a novel target for therapeutic intervention in pathological conditions mediated by
defects in osteoclast production through cell-cell fusion.

Yeast Cell Polarity

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Cell polarity kinases control cell shape through the RNA-binding protein Sts5
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A long-standing question in cell biology is how cells achieve a polarized growth state that can be
modified by environmental stress. Rod-shaped fission yeast cells elongate in a highly polarized manner
due in part to protein kinases that localize to growing cell tips. In screening the localization of these
kinases under stress conditions, we discovered that the p21-activated kinase Pak1 rapidly moved into
RNP (ribonucleoprotein) stress granules during glucose starvation. Through a phosphoproteomic screen
for Pak1 substrates, we discovered that Pak1 directly phosphorylates the intrinsically disordered region
(IDR) of Sts5, an RNA-binding protein found in RNP stress granules. In sts5Δ mutant cells, Pak1 did not
relocate to stress granules during glucose starvation. Interestingly, in glucose-rich conditions, Pak1
prevented both the clustering and stress granule localization of Sts5. Thus, Pak1 and Sts5 regulate each
other, but the direction of regulation depends on glucose availability. In glucose-rich media, mutation of
a single Pak1-dependent phosphorylation site on Sts5 leads to constitutive RNP granule localization of
Sts5 and polarized growth defects. A second cell polarity kinase, Orb6, was previously shown to
phosphorylate a different residue in the Sts5 IDR. We found that mutation of both phosphorylation sites
on Sts5 caused severe defects in cell growth rate, morphology, and septation. These defects were linked
to dysregulation of Sts5-bound RNAs at RNP granules. These findings provide a new mechanism for cell
polarity establishment by two conserved protein kinases acting through RNPs, and an unexpected
bidirectionality of Pak1-RNP regulation according to nutrient conditions.
Adaptability and evolution of the cell polarization machinery in budding yeast

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How can self-organized function evolve, adapt to perturbations, and acquire new sub-functions? To make progress towards answering these fundamental questions, we analyze, as a concrete example, the cell polarity machinery of Saccharomyces cerevisiae, which is essential for cell growth and division. Recently, an experimental evolution study demonstrated that this polarization machinery can recover quickly and reproducibly from the knockout of one of its key components. Using a combination of modeling, conceptual theory, and experiments, we identified and characterized the underlying rescue pathway. It is based on a latent polarization mechanism that operates on a different subset of proteins than the wild-type mechanism. Based on the mechanistic insights we gain from dissecting the wild-type and rescue mechanism, we hypothesize how scaffold proteins, by introducing new connections in an existing network, can increase the redundancy of mechanisms and thus increase the evolvability of other network components.

Fission yeast polycystin Pkd2p channel promotes cell growth through interacting with SIN and MOR pathways

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The regulation of cell morphogenesis is essential throughout cell cycle. The rod-shaped unicellular fission yeast cells grow from their tips during interphase but maintain a constant cell length throughout mitosis without any tip expansion. We recently identified pkd2, the fission yeast homologue of polycystin, as essential for both cytokinesis and cell growth. Polycystins are a family of evolutionally conserved cation channels, found in both unicellular and multicellular eukaryotes. Mutations of human polycystins lead to Autosomal Dominant Polycystic Kidney Disorder (ADPKD), a renal disorder associated with the over-growth of kidney cysts. However, it remains unclear how polycystins regulate cell growth. Here we examined the function of yeast Pkd2p through characterizing a novel temperature-sensitive mutant of pkd2. At restrictive temperature, the pkd2-B42 mutant cells failed to grow from their tips, preventing the mitotic entry. In addition, the width of these mutant cells increased by 17%. We examined the genetic interaction between pkd2-B42 with more than thirty fission yeast mutants. We discovered that pkd2 mutant strongly interacted with the mutants of two essential pathways SIN and MOR, respectively. The pkd2 mutation partially rescued the mutants of Septation Initiation network (SIN) pathway, which is essential for cell morphogenesis during cytokinesis. The pkd2 mutant specifically prevented lysis of the SIN mutant cells but did not rescue their defect in septation. Mutation of pkd2 did not alter either the localization or the activity of SIN proteins, suggesting that Pkd2p does not regulate the SIN pathway directly. Conversely, pkd2-B42 exacerbated the growth defect of the mutants of Morphogenesis Orb6 Related (MOR) network which is essential for the interphase cell growth. MOR mutants failed the tip growth but they were able to expand from their sides. In comparison, mutation of pkd2 further inhibited the lateral growth of the MOR mutants, stalling cell growth completely. About
55% of the double mutants of \textit{pkd2} and MOR mutant cells shrank, becoming shorter and narrower, suggesting a defect in the maintenance of turgor pressure. In summary, we conclude that the fission yeast Pkd2p promote cell growth throughout the cell cycle, synthesizing with the MOR pathway during interphase and antagonizing the SIN pathway during cell division.

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\textbf{Cell-cycle-dependent cues temporally regulate Cdc42 activity at growth sites in fission yeast}

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Cells undergo polarized growth to acquire a shape that promotes function. In fission yeast, polarized cell growth is activated by the Morphogenesis Orb6 (MOR) pathway and the small GTPase Cdc42. After cell division, the MOR pathway first promotes cell separation, the final step in cytokinesis, and then promotes polarized growth at the cell ends. It is unclear how the ends initiate growth after the cells undergo separation. It is plausible that the MOR pathway activates end growth only if it successfully enables cell separation. To test this, we developed a system whereby we delay cytokinesis, while mitosis progresses, via a temporary Latrunculin A (LatA) treatment. Cells treated with LatA during mitosis, when allowed to recover, initiate old end growth without cell separation. We call this the PrESS phenotype - polar elongation sans cell separation. PrESS cells show Cdc42 re-activation at the ends often before the completion of cytokinesis, showing that growth occurs independently of cell separation. Additionally, we find that Cdc42 regulators, Cdc42 itself, and trafficking machinery are siphoned away from the cell middle and appear at the cell ends, suggesting a competition between the cell middle and the ends. This competition leads to cell separation failure since the requisite digestive enzymes are not secreted to promote septum digestion. To identify how initiation of Cdc42 activation at cell ends is regulated after cell division, we performed a candidate screen with Cdc42 regulators. We find that loss of the Cdc42 inactivator Rga4 increases the frequency of the PrESS phenotype. Moreover, we show that the distribution of Rga4 along the cortex changes according to the cell-cycle stage, displaying a punctate appearance mostly relegated to the cell sides during G2, and a more diffuse appearance that extends to the cell ends during mitosis. We hypothesized that growth at cell ends requires simultaneous MOR pathway activation, which promotes protein synthesis, and Rga4 removal from the ends after mitosis. To test this, we constitutively activated the MOR pathway in an \textit{rga4} deletion mutant. Cells constitutively activating the MOR pathway often lyse due to premature synthesis and delivery of digestive enzymes to the division site. We find that deletion of \textit{rga4} in these cells enhances cell end growth while preventing cell separation and rescuing lysis, thus recapitulating the PrESS phenotype. Therefore, we propose that Rga4 is removed from the cell ends during cell separation in a cell-cycle-dependent manner, allowing Cdc42 activation and growth at these ends.

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\textbf{Exploratory polarization facilitates mating partner selection in \textit{Saccharomyces cerevisiae}}

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Yeast decode pheromone gradients to locate mating partners, providing a model for chemotropism. How yeast polarize toward a single partner in crowded environments is unclear. Initially, cells often polarize in unproductive directions, but then they relocate the polarity site until two partners’ polarity sites align, whereupon the cells “commit” to each other by stabilizing polarity to promote fusion. Here we address the role of the early mobile polarity sites. We found that commitment by either partner failed if just one partner was defective in generating, orienting, or stabilizing its mobile polarity sites. Mobile polarity sites were enriched for pheromone receptors and G proteins, and we suggest that such sites engage in an exploratory search of the local pheromone landscape, stabilizing only when they detect elevated pheromone levels. Mobile polarity sites were also enriched for pheromone secretion factors, and simulations suggest that only focal secretion at polarity sites would produce high pheromone concentrations at the partner’s polarity site, triggering commitment.

**Tuesday, December 15, 2020, 11:00 am**

*Actin and Actin-Binding Proteins in Mechanobiology and Engineering 1*

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**Contractile force reveals new insights about cell recovery after electroporation**

*P. Graybill, A. Jana, R. Kapania, A. Nain, R. Davalos; Virginia Tech, Blacksburg, VA.*

Electrical fields are ubiquitous in how cells function, communicate and survive. External electric fields, applied as short high-voltage pulses, can disrupt the cell membrane and increase membrane permeability through a process known as electroporation. Cellular recovery after electroporation is difficult to monitor and is typically limited to membrane permeability markers or cytoskeleton stains. Furthermore, electroporation is usually studied in cells that are either in suspension (no adhesions) or attached to non-physiological 2D flat substrates (isotropic distribution of adhesions). Very little is known about cell response to pulsed electric fields when cells are attached to extracellular matrix (ECM) mimicking fibers that cause adhesions to be localized to poles. Here we measured the cell shape and contractile response of single cells adherent to ECM-mimicking fibers after application of high-voltage pulsed electric fields. We constructed nanofiber scaffolds using our non-electrospinning spinneret based tunable engineered parameters (STEP) platform. Using nanonet force microscopy (NFM), we measure the contractile forces of cells as they attach to and deflect the suspended fibers. Sublethal, 100µs duration electrical pulses permeabilized the cell membrane and resulted in rapid and significant loss of contractile forces in both healthy and cancerous cells. We show that the magnitude of mechanical disruption is dependent on the applied voltage and cell orientation, and that mechanical recovery occurs within 1-3 hours after treatment. We found that contractile force is a more sensitive and dynamic metric of cell recovery than cell shape, and that the magnitude of cell rounding after electroporation is cell line dependent. Unexpectedly, we found that cells can display a multi-stage response that includes a cell-rounding stage, a biphasic force stage, and a spreading stage. In glioblastoma cells, the force signature during the bi-phasic response correlates with the strength of electrical pulse, coincides with cytoskeletal disruption-driven blebbing, and is most pronounced when the electric field is applied along the cell length. Altogether, our method for monitoring cell contractile forces provides new insight into cell recovery after electroporation, and may lead to new opportunities in electroporation applications such as genetic engineering, gene therapy and molecular medicine.
Development of a switchable actin binder
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The actin cytoskeleton plays a critical role in diverse cell processes, including formation of tight junctions between epithelial cells and phagocytosis of target particles by immune cells. Regulation of these distinct processes within the shared cytoplasm of a cell requires a broad set of actin-binding proteins that organize actin filaments into specific structures through tethering, crosslinking, and nucleating activities. We developed a switchable actin binder that binds to actin only when a cell-permeable small molecule is administered, to investigate actin tethering in live cells. The switch comprises three fused protein domains - an actin-binding motif and two flanking domains that heterodimerize to block actin binding. For the actin-binding motif, we chose the actin binding site (ABS) of ZO-1 and sandwiched it between truncated Bcl-xL and the modified peptide, BH3, whose binding can be disrupted by the small molecule A-1155463. We confirmed that when the switchable actin binder is expressed in cells and the small molecule is administered, the switchable actin binder colocalizes with actin. Furthermore, we show that the switchable actin binder is tunable; with increasing concentration of the small molecule, there is increasing colocalization of the switchable actin binder with actin. To demonstrate functionality of the probe, we engineered ZO-1, an adapter protein that links actin filaments with tight junction proteins, by replacing the ABS of ZO-1 with the switchable actin binder. We found that when actin binding was inhibited in ZO-1, epithelial barrier function was reduced based on measurements of trans-epithelial electrical resistance. By administering A-1155463 to open the switchable actin binder within ZO-1, barrier function significantly increased. As a second demonstration of the switchable actin binder, we incorporated a CAAX motif that localized the switch to the plasma membrane and expressed it in macrophages. We found that when A-1155463 was administered, phagocytosis of antibody-coated targets decreased, filopodia were suppressed, and Fc receptor enrichment at the target site required more time. As indicated by these results, a switchable actin binder that controls when, where, and how much actin binding occurs in live cells has the potential to be a useful and versatile tool for investigating the role of actin networks in cells.

Shootin1b as a clutch molecule during cell-cell contact formation in epithelial cells
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Epithelial cell-cell adhesion exhibits dynamic behavior that is deemed necessary for various biological processes such as cell motility, cell arrangement and tissue folding. To form and maintain cell-cell adhesion, cells require cadherins and actin cytoskeleton. The linkage between cadherins and actin filaments (F-actins) is indirect and is mediated by β- and α-catenins. Epithelial cell extends lamellipodia-like protrusions underneath adjacent cells during cell-cell adhesion formations. These overlapping protrusions then slides rearward to form matured cell-cell contact. How the interface between the two-contacting membranes is regulated is not clearly understood. Here, we provide evidence that shootin1b functions as a clutch molecule in lamellipodia-like active protrusions of overlapping epithelial cells.
during cell-cell adhesion formation. Clutch molecules mediate dynamic mechanical coupling between polymerizing F-actins that undergo retrograde flow and cell adhesion complex. In migrating olfactory neurons, shootin1b accumulates at the growth cones of their leading process and couples F-actin retrograde flow, via cortactin and L1-CAM, with adhesive substrate, thereby generating traction force on the substrate. We show that shootin1b co-localizes with E-cadherin and F-actin at the cell-cell contact sites of cultured epithelial cells. By co-immunoprecipitation, in vitro binding assay and co-sedimentation assay, we demonstrate that shootin1b mediates the linkage between E-cadherin and the actin cytoskeleton. Shootin1b knockout perturbed the junctional accumulation of E-cadherin and F-actin in EpH4 mammary gland epithelial cells. In addition, small intestines tissue derived from shootin1-knockout mice exhibited elongated epithelial cells and abnormal nuclear positioning. Furthermore, loss of shootin1b decelerated the formation of cell-cell contact in calcium switch assays. To understand the mechanism on how shootin1b mediates cell-cell adhesion in these cells, we performed fluorescent speckle-imaging of shootin1b, F-actin and E-cadherin at the active protrusions between the neighboring cells. Our data demonstrate that shootin1b undergo retrograde movement with F-actin and E-cadherin on the overlapping protrusions of two-contacting cells. These data suggest that shootin1b functions as a clutch molecule on active protrusion of overlapping epithelial cells during cell-cell contact formation.

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**A parallelized platform for visualizing mechanical regulation of F-actin**

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Actin filaments (F-actin) can act as mechanical sensors, engaging specific actin binding proteins (ABPs) in a force-dependent manner to transduce mechanical stimuli into biochemical signaling pathways. While several force-modulated F-actin-ABP interactions have recently been identified, many open mechanistic questions remain. These include establishing the magnitudes and types of forces (e.g. tension, compression, and torsion) mediating mechanical regulation, as well as investigating how forces coordinately regulate higher-order actin networks and individual filaments. Here we focus on dissecting the impact of forces generated by myosin motor proteins. We have devised an approach using micro-patterned stripes of surface-immobilized myosin V to apply tensile forces to many actin filaments in parallel. We conjugate two filaments in a head-to-head arrangement using an engineered, covalently dimerized barbed-end capping protein complex, which we collectively term a “paired filament complex” (PFC). In the presence of active force generation, tensed PFCs are captured between myosin V stripes, facilitating visualization of mechanical regulation with Total Internal Reflection Fluorescence (TIRF) microscopy. As compared to single-molecule approaches, e.g. optical and magnetic traps, our technique allows simultaneous analysis of hundreds of distinguishable PFCs under tension at different positions in the microscopic field of view over many minutes. We find a subset of PFCs spontaneously form intertwined complexes featuring “arms” composed of different numbers of actin filaments, facilitating simultaneous dissection of the contributions of binding and bundling to force-dependent F-actin interactions. We are currently working to incorporate fluorescent tension reporters, as well as other linker components, to establish a modular molecular toolkit for probing mechanical regulation.
**Myosin II and Arp2/3 crosstalk governs intracellular hydraulic pressure and lamellipodia formation**

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Human fibroblasts can switch between lamellipodia-dependent and -independent migration mechanisms on 2D surfaces and in 3D matrices. RhoA GTPase activity governs the switch from low-pressure lamellipodia to high-pressure lobopodia in response to the physical structure of the 3D matrix. Inhibiting actomyosin contractility in these cells reduces intracellular pressure and reverts lobopodia to lamellipodial protrusions via an unknown mechanism. To test the hypothesis that high pressure physically prevents lamellipodia formation, we manipulated pressure by activating RhoA or changing the osmolarity of the extracellular environment and imaged cell protrusions. We find RhoA activity inhibits Rac1-mediated lamellipodia formation through two distinct pathways. First, RhoA boosts intracellular pressure by increasing actomyosin contractility and water influx but acts upstream of Rac1 to inhibit lamellipodia formation. Increasing osmotic pressure revealed a second RhoA pathway which acts through non-muscle myosin II (NMII) to disrupt lamellipodia downstream of Rac1 and elevate pressure. Interestingly, Arp2/3 inhibition triggered a NMII-dependent increase in intracellular pressure, along with lamellipodia disruption. Together, these results suggest that actomyosin contractility and water influx are coordinated to maintain cell volume and increase intracellular pressure, and RhoA signaling can inhibit lamellipodia formation via two distinct pathways in high-pressure cells.

**Actin/tropomyosin filaments derived from the Arp2/3 network mediate focal adhesion maturation and mechanosensitivity of cell proliferation**

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We identified a specific actin/tropomyosin filament population derived from severing of the Arp2/3 branched actin network present in the lamellipodium of migrating mouse embryo fibroblasts. This filament population is composed of co-polymers of tropomyosins Tpm1.8/1.9 and actin that specifically anchor to focal adhesions and support their maturation. Tpm1.8/1.9/actin filaments are required to recruit the adhesion proteins paxillin and zyxin to focal adhesion sites. Furthermore, both knock-down and knock-out of this actin/tropomyosin filament population causes significantly fewer focal adhesions, disrupted adhesion maturation, and altered paxillin/phospho-paxillin levels. Phospho-paxillin, the main driver of adhesion turnover, is increased by 15-fold in the absence of these filaments, whereas, total paxillin and zyxin, key components for adhesion maturation, are reduced by 30% and 90%, respectively. This adhesion defect leads to cell detachment and anoikis on stiff substrates. Notably, this phenotype is reversed when cells are grown on soft substrates. This defective mechanosensing phenotype is compatible with a role for Tpm1.8/1.9/actin filaments in the formation of contractile units (CUs). Therefore, we tested whether the lack of this these filaments affects the levels of three key components...
of CUs, α-actinin, Tpm2.1 and Myosin-IIa. We find that this subgroup of actin filaments is required for α-actinin crosslinking, and in its absence the formation of α-actinin bundles across the lamella is greatly reduced. When α-actinin recruitment is hindered, CUs disassemble and rigidity sensing fails, leading to increased cell proliferation on soft substrates. Furthermore, the levels of the three components are significantly reduced in the absence of these actin/tropomyosin filaments; whereas, the levels of all other tropomyosin isoforms that are not present in CUs remain unchanged. We conclude that a Tpm1.8/1.9/actin filament population originating from severing of the Arp2/3-actin network at the cell’s leading edge is essential to anchor focal adhesions and promote adhesion assembly and maturation. This filament population regulates rigidity sensing via CU assembly and inhibits cell proliferation on soft substrates, and its downregulation upon cell transformation may contribute to the growth of human cancer.

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**Actin polymerization controlled by mechanical force protects the cytoskeleton from damage and facilitates stress fiber repair**

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Cells are truly the ultimate “smart material” fine-tuning their mechanical properties to match the mechanical demands of their environments. Such plasticity of cellular mechanics underlines a wide range of cell and tissue behaviors such as allowing cells to migrate through narrow spaces, to resist blood shear forces, and to reinforce force bearing tissues. Moreover, the plasticity of cell mechanics, which relies for the most part on the regulation of the structure and dynamics of actomyosin cytoskeleton, plays a protective role and safeguards the cell against mechanical damage. Yet, the molecular players implicated in adaptive changes in cell mechanics are unknown. By combining live-cell imaging and pharmacological manipulations, we assessed how suppression of major actin elongation factors affects the dynamics of actin polymerization at integrin-based focal adhesions, force bearing linkages between the actin cytoskeleton and extracellular matrix. We found that inhibiting formins but not Arp2/3, significantly decreased the rate of actin polymerization at focal adhesions. By depleting individual formins with siRNA, we identified mDia1 as the major actin elongation factor at focal adhesions. To determine whether Dia1 activity is regulated by mechanical forces, we assessed the effect of myosin inhibition on the rate of actin polymerization in the control and mDia1 depleted cells. Analysis of control cells has revealed a dose-dependent decrease in actin polymerization rate at focal adhesions as myosin contractility was suppressed by ~90%. In contrast, the rate of actin polymerization in mDia1 depleted cells was not affected by myosin inhibition, suggesting that myosin-generated tension modulates mDia1 activity. Furthermore, by using a super-localization microscopy we showed that actin polymerization at focal adhesions exhibits pulsatile dynamics where the spikes of Dia1 activity are triggered by mechanical force. By combining mathematical modeling and laser nano-surgery, we showed that suppression of force-dependent actin polymerization at focal adhesions results in two-fold increase in mechanical tension on the stress fibers. To assess how this increase in mechanical tension affects the integrity and repair of actin cytoskeleton we used flashes of the LIM domain protein zyxin as a marker for cytoskeleton damage. We found that suppression of actin polymerization at focal adhesions increases the magnitude of spontaneous stress fiber damage and decreases the efficiency of
zyxin-mediated stress fiber repair. As the actin cytoskeleton as a whole is subject to complex mechanical constraints in living cells, these results demonstrate the key role of mDia1 in fine-tuning actin organization to prevent cytoskeleton damage.

**Chaperones, Protein Folding, and Quality Control: Quality Control & Disease**

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**A High Frequency Mutation-Independent Mechanism of Drug Resistance**

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The rapid emergence of drug resistance is a major cause of treatment failures. The most well studied mechanisms of drug resistance, from tumors to pathogens, are driven by mutations, which often alter drug targets or upregulate multidrug transporters. Here, using the leading human fungal pathogen *Candida albicans* as a model, we describe an entirely different epigenetic mechanism that promotes the development of high frequency drug resistance. This mode of drug resistance, which we term ‘pararesistance,’ is both heritable and reversible. Exposure to a low dose of fluconazole, the most widely prescribed antifungal, gives rise to a stable subpopulation of pararesistant resistant *C. albicans* cells (1–10% of a culture). This high frequency, along with patterns of appearance in Luria-Delbruck fluctuation tests, high reversion frequency, and negligible fitness cost in the absence of drug distinguish pararesistance from mutation-based resistance mechanisms. Furthermore, inhibition of Hsp90 with geldanamycin or radicicol significantly increases the induction frequency, while exposure to low levels of the protein denaturant guanidine hydrochloride blocks the induction of pararesistance. Likewise, although pararesistance can be stably maintained for >100 generations in the absence of drug, its loss is accelerated by the transient guanidine hydrochloride exposure. Our results suggest that the induction and maintenance of pararesistance are modulated by components of the proteostasis network. RNA-sequencing analysis shows that pararesistant isolates have very different transcriptional profiles from susceptible isolates and, strikingly, exhibit little additional response when exposed to fluconazole. In addition, multidrug transporters such as *CDR1* are constitutively upregulated in pararesistant cells. Consistent with this finding, pararesistant isolates show increased efflux, resulting in decreased accumulation of Cdr1 substrate rhodamine 6G. Finally, turning to a clinical isolate of *C. albicans* from the sputum of a patient with recurrent candidiasis and a history of antifungal therapy, we uncovered heterogeneity in fluconazole resistance consistent with pararesistance. Our work defines a pervasive mutation-independent mechanism that can spark the rapid evolution of drug resistance and may underlie recurrent episodes of candidiasis in the clinic.

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**Elucidating the quality control pathway of KCC2, a critical synchronizer of neuronal development**

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Neurodevelopmental disorders are a debilitating category of diseases that initiate in gestation when synaptogenesis is crucial and reliant upon electrochemical ion gradients across the neuronal plasma...
membrane. For example, the K⁺/Cl⁻ co-transporter 2 (KCC2) regulates γ-aminobutyric acid (GABA) neurotransmission and has been linked to epilepsy, schizophrenia, and autism spectrum disorders. Importantly, KCC2 is a complex multimeric membrane protein that exhibits reduced cell surface expression and/or activity when disease-associated mutations are present. This observation is indicative of an underlying instability in the KCC2 protein, potentially making it highly susceptible to cellular quality control pathways that encourage proper folding and/or target misfolded proteins for degradation. Despite its critical role in neurodevelopment and disease relevance, little research has been done to define the mechanisms that modulate KCC2 protein stability and cell surface expression. The objective of this study is to elucidate the pathways that regulate KCC2 folding and degradation to better understand its role in the pathogenesis of neurodevelopmental disorders. To this end, cycloheximide chase assays were conducted in a new yeast expression system for wild-type KCC2 and stability was assessed over time after protein synthesis was arrested. Western blot analysis revealed that KCC2 is highly unstable. To determine which quality control pathway is responsible for the degradation of KCC2, cells were first treated with MG132, an inhibitor of the proteasome. Because MG132 treatment significantly stabilized KCC2, we propose that the transporter is subject to endoplasmic reticulum-associated degradation (ERAD). This hypothesis is supported by an observed increase in KCC2 stability when protein stability was measured in strains lacking two ERAD-requiring E3 ubiquitin ligases. KCC2 stabilization is also observed when yeast expressed a dominant negative version of Otu1, an ERAD deubiquitinase whose levels are altered in synaptosomes isolated from patients with schizophrenia. To validate these findings in a mammalian model, cycloheximide chase assays were conducted in HEK293 cells in the presence or absence of MG132 treatment. Preliminary data suggest that the ER-resident fraction of wild-type KCC2 is unstable and rapidly targeted for proteasome-dependent degradation. Together, these results confirm the underlying instability of KCC2 in two model systems. This study represents the first steps toward elucidating the quality control pathways that regulate KCC2 degradation and suggest that the ERAD of disease-causing mutant forms of KCC2 might contribute to disease onset.

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Characterization of gain- and loss-of-function variants in the renal potassium channel, ROMK

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Elevated blood pressure (hypertension) affects one billion people worldwide and is the most common risk factor for cardiovascular disease. Despite the high heritability of the condition, the relative contributions of known and as yet undiscovered genetic factors have yet to be fully defined. Regulators of hypertension include salt-handling transporters in the kidney, among which the renal outer medullary K⁺ (ROMK) channel is of particular interest. Recent data indicate that unstable mutant forms of ROMK are prone to premature degradation, which give rise to Bartter syndrome Type II, a renal salt-wasting disease [1]. To systematically assess the impact of all possible ROMK missense variants, we then utilized both a computational algorithm, Rhapsody, that predicts the effects of missense variants based on sequence and structural dynamics, as well as experimental assays that measure ROMK activity in yeast. We found that Rhapsody correctly predicted a majority of deleterious and Bartter syndrome-associated mutations in ROMK [2]. In this era of ever-expanding human genomic databases, the consolidation of
computational and experimental approaches to systematically evaluate the functional impact of human variants is vital and will accelerate the identification of disease-causing mutations in any protein. Although loss-of-function mutations give rise to Bartter syndrome, heterozygotes with these same mutations are protected from hypertension. Therefore, we hypothesized that gain-of-function (GOF) mutations in ROMK that increase potassium flux will predispose individuals to hypertension. To test this hypothesis, we utilized an unbiased genetic screen in yeast lacking two major K+ transporters, so growth is restricted on low K+. We then expressed ROMK in the strain and screened for clones containing random mutations in ROMK that exhibited better growth on low K+. By utilizing both this unbiased screen and a candidate-based approach, we identified four potential GOF mutations, including one that exists in the human TOPMed database. Some of the mutants exhibit increased stability in yeast or increased channel conductance in *Xenopus* oocytes, an established model to study ion conductance, and our data are consistent with the observed GOF phenotype. Current studies are characterizing how the mutations affect protein trafficking and assembly in both yeast and mammalian cell models. Ultimately, our findings will be translated into murine models in which blood pressure regulation can be directly assessed. 1.O’Donnell, B.M, et al. J Biol Chem, 2017. 292(31): p. 12813-12827. 2.Ponzoni, L. and Nguyen, N. H., et al. PLoS Comput Biol, 2020. 16(4): p. e1007749.

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**Utilizing endoplasmic reticulum-associated substrates to uncover novel mammalian disaggregases**  
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Neurodegenerative diseases afflict over 5 million Americans. A large percentage of these conditions are caused by the presence of misfolded proteins and protein aggregates that disrupt proteostasis, which maintains protein function and “quality control” in the cell. Normally, cells can handle small aggregates through the action of molecular chaperone assemblies and protein degradation pathways. However, highly stable aggregates irreversibly disrupt proteostasis and trigger disease onset. In contrast to human cells, the chaperone Hsp104 can resolve highly stable aggregates in yeast. Problematically, humans lack Hsp104. Therefore, we hypothesize that metazoan cells have developed alternative machinery to resolve stable protein aggregates. To address this hypothesis, we developed multiple endoplasmic reticulum (ER) localized substrates that have aggregation-prone cytosolic motifs. For example, GD* is a substrate consisting of the first two transmembrane domains of yeast Ste6 fused to a temperature sensitive mutant of Ubc9. GD* was chosen as a model substrate to characterize mammalian disaggregases because it is targeted by Hsp104 in yeast (1). Additional substrates with mammalian protein-derived membrane anchors and aggregation-prone and amyloid-like motifs were also developed. Because misfolded proteins at the ER can be targeted by multiple degradation pathways, including ER associated degradation (ERAD) or autophagy, we began by evaluating these substrates in HEK293H cells with cycloheximide chase and detergent fractionation assays. We discovered that GD* largely depended on the proteasome for degradation and showed decreased solubility when cells were incubated at elevated temperatures. In contrast, a control substrate lacking the temperature sensitive mutation, GD, was more soluble than its aggregation-prone counterpart. These results suggest that protein substrates face differential fates depending on their aggregation state and may rely on different sets of chaperones for ERAD targeting in human cells. In the future, we will identify proteins associated with our substrates via crosslinking and mass spectrometry, and assess identified partners for disaggregate activity. (1) Preston et al., Mol Cell (2018) 70(2):242-253
Mutational mapping of a protein self-assembly landscape in living cells
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Many proteins contain low complexity domains (LCD), which lack well-folded structures in their native states. LCD-containing proteins are the major component of proteinaceous aggregates found in multiple neurodegenerative diseases. One of the most prominent disease is TDP-43 in amyotrophic lateral sclerosis (ALS). TDP-43 has become a model LCD protein, in part due to its ability to form multiple different types of self- assemblies, ranging from highly ordered amyloid-like aggregates to disordered condensates and gels. TDP-43 LCD is composed of two glycine-aromatic-serine-rich (GaroS) regions, one Q/N rich region and one short hydrophobic patch that forms alpha-helices in a cooperative fashion. Structural studies \textit{in vitro} have revealed several amyloid cores for TDP-43. However, the extent to which any of these assembly modalities occur in cellular contexts, their kinetic relationships to one another, and their relevance to TDP-43 function and dysfunction all remain unclear. Here, we combined high-throughput DAmFRET, a quantitative fluorescence-based reporter of protein self-assembly, with rational mutagenesis and all atom simulations to systematically map the energy landscape of TDP-43 LCD self-association in a cellular context. We observed four distinct types of TDP-43 LCD self-assembly in living cells -- soluble helix-mediated oligomers, liquid droplets, and two discrete forms of TDP-43 amyloid. We dissected the structural and kinetic relationships between each type of self-assembly. Our data reveal a surprising competition between alpha-helicity and condensation, and that these two forms of assembly lead to different types of amyloid. This is to our knowledge the most comprehensive analysis of the pathways of amyloid formation by a single protein in living cells.

Tuning of the Unfolded Protein Response by BiP AMPylation is required for photoreceptor plasticity
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In response to environmental, developmental, and pathological stressors, cells engage homeostatic pathways to maintain their function. Among these pathways, the Unfolded Protein Response (UPR) protects cells from the accumulation of misfolded proteins in the ER. The activity of the major endoplasmic reticulum chaperone and UPR master regulator BiP is regulated by Fic-mediated AMPylation during resting states and Fic-mediated deAMPylation during times of stress. In this study, we aimed to understand the consequences of this regulation of BiP in the Drosophila visual system. After 72 hours of constant light, photoreceptors of fic-null and AMPylation resistant BiPT366A mutants, but not wild-type flies, display loss of synaptic function, disintegration of rhabdomes, and excessive activation of ER stress reporters. Strikingly, this phenotype is partially reversible as photoreceptors of these mutant flies recover much of their structure and function within 72 hours once returned to a standard light:dark cycle. These findings show that Fic-mediated AMPylation of BiP is required for
neurons to adapt to transient stress demands. In addition, we show that excessive AMPylation by a constitutively-AMPylyating FicE247G mutant is lethal in Drosophila. This lethality is cell autonomous as directed expression of the mutant FicE247G to the fly eye does not kill the fly but rather results in a rough and reduced eye. Lethality and eye phenotypes are rescued by the deAMPylation activity of wild-type Fic, consistent with Fic acting as a deAMPylation enzyme. Furthermore, lethality is also rescued by the expression of AMPylation-resistant BiPT366A, indicating unregulated AMPylation of BiP is lethal. Taken together, our data supports a model by which Fic mediated BiP AMPylation changes the threshold at which an Unfolded Protein Response occurs in these cells which has physiological consequences to the fly visual system.

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**HYPE-mediated AMPylation as a Novel Therapeutic Target for Parkinson's Disease**

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A major hallmark of Parkinson’s disease (PD) is the deposition of the intrinsically disordered protein α-synuclein (αSyn) into intracellular inclusions termed Lewy bodies. HYPE—the sole human representative of a conserved family of adenyllyltransferase enzymes—has been shown to covalently modify (AMPylate) αSyn in vitro. Remarkably, HYPE-mediated AMPylation ameliorates many of the neurotoxic phenotypes of αSyn implicated in the progression of PD, such as αSyn fibrillation and membrane permeability. These potentially cytoprotective phenomena conferred by HYPE’s adenyllyltransferase activity make it an attractive therapeutic target. Unfortunately, wild-type HYPE is intrinsically inhibited, showing only basal AMPylation levels relative to a constitutively active mutant (E234G-HYPE). To this end, we set out to screen both FDA-approved and proprietary small-molecule compound libraries towards the identification of novel manipulators of HYPE AMPylation Employing the fluorescence polarization of an ATP analog fluorophore—Fl-ATP—we developed and optimized an efficient, robust assay which monitors HYPE autoAMPylation and is amenable to automated, high-throughput processing of diverse chemical libraries. Challenging our pilot screen with compounds from the LOPAC, Spectrum, MEGx, and NATx libraries yielded 0.3% and 1% hit rates for HYPE activators and inhibitors, respectively. Further, these hits were assessed for dose-dependency and validated via orthogonal biochemical AMPylation assays. We thus present a high-quality HTS assay suitable for tracking HYPE’s enzymatic activity, and the first small molecule manipulators of HYPE-promoted autoAMPylation. Challenging neuronal cell culture models of PD with these hits provides molecular insights into αSyn-induced neurotoxicity, and paves the path for novel therapeutic strategies in combating PD.

**Chromosome Organization: Mammalian 1**

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**Chromosome-Loss Reporter shows Mechanical Confinement can lead to Chromosome Misseggregation.**

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Live Cell Chromosome Loss Reporter shows Mechanical Confinement associates with Chromosome Mis-segregation Cell division within solid tumors is confined by a stiff niche of adjacent cells and extracellular matrix, and such confinement has been reported to cause chromosome mis-segregation. We find confinement chambers can also cause cell death, and so we developed a chromosome-loss-reporter to identify viable cells that have mis-segregated chromosomes. Mitotic cells under in vitro confinement exhibit more abnormal division and more fluorescence-null cells, compared to 2D control cultures, for both cancer and normal cell types. Reporter cells grown as tumors in mice also show more fluorescence-null cells than 2D cultures, consistent with the stress in vivo causing chromosome mis-segregation.

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Micronuclei in Kif18a knockout mice form stable micronuclear envelopes and do not drive tumorigenesis

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Micronuclei, whole or fragmented chromosomes which are spatially separated from the main nucleus, are strongly associated with genomic instability and have been identified as drivers of tumorigenesis. Micronuclei are shown to contribute to genomic instability through two non-mutually exclusive pathways: 1) cells that enter cell division with a micronucleus can incur severe, localized DNA rearrangements to the micronucleated chromosome [1,2], and 2) following the loss of micronuclear envelope integrity, exposed chromosomes can become damaged in the cell’s cytoplasm [3,4]. Paradoxically, Kif18a mutant mice, which produce micronuclei due to unaligned chromosomes in vivo, do not develop spontaneous tumors, suggesting that not all micronuclei contribute similarly to genomic instability and cancer [5,6]. We report here that micronuclei in Kif18a mutant mice form stable nuclear envelopes. Challenging Kif18a mutant mice via deletion of the Trp53 gene led to formation of thymic lymphoma with elevated levels of micronuclei. However, loss of Kif18a had modest or no effect on survival of Trp53 homozygotes and heterozygotes, respectively. To further explore micronuclear envelope stability in KIF18A KO cells, we compared micronuclei induced via different insults in vitro. Micronuclei in KIF18A KO cells form stable nuclear envelopes characterized by increased recruitment of core and non-core nuclear envelope components and successful expansion of decondensing chromatin within the micronucleus compared to those induced by microtubule drug washout or exposure to radiation. As a result of this stability, these KIF18A KO micronuclei rupture infrequently (19% rupture), compared to micronuclei induced by microtubule drug washout (58% rupture; p < 0.001) or exposure to radiation (35% rupture; p < 0.01). We also observed that late-lagging chromosomes, which lead to micronucleus formation, were positioned closer to the main chromatin masses, and further from the central spindle, in KIF18A KO cells. Our studies provide in vivo support to models proposing that the subcellular location of late lagging chromosomes strongly correlated with micronuclear fate [4,7] and suggest that not all micronuclei equally impact tumorigenesis. 1. Crasta et al., 2012, Nature. 2. Zhang et al., 2015, Nature. 3. Hatch et al., 2013, Cell. 4. Liu et al., 2018, Nature. 5. Zhu et al., 2013, Biochemical and Biophysical Research Communications. 6. Fonseca et al., 2019, Journal of Cell Biology. 7. Alfonso et al., 2014, Science.
Misaligned chromosomes are a primary cause of chromosomal instability in breast cancer and are poorly immunostimulatory

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Chromosomal instability (CIN), the persistent gain and loss of chromosomes during mitosis, is a common characteristic of human cancers that contributes to tumor heterogeneity. Though multiple mechanisms can produce CIN, lagging chromosomes generated from merotelic attachments are the major cause of CIN in cell lines, and are therefore expected to predominate in cancer. To assess the source of mitotic instability in breast cancer, we used a primary tumor tissue microarray, a matched cohort of primary and metastatic samples and patient derived organoids from primary breast cancer to quantify mitotic defects. Surprisingly, in all contexts misaligned chromosomes are more common than lagging chromosomes. Metastatic breast cancers exhibit higher rates of mitotic defects than matched primary cancers, primarily due to an increase in misaligned chromosomes. To determine which type of chromosome segregation error(s) were the predominant source of CIN, we correlated the incidence of mitotic defects with CIN in primary breast cancer as measured by 6-chromosome interphase FISH. Misaligned chromosomes strongly correlated with CIN, while lagging chromosomes and chromosome bridges did not, supporting misaligned chromosomes as a major cause of CIN in breast cancer. Misaligned chromosomes result in micronuclei less frequently than lagging and bridge chromosomes. Since micronuclei commonly activate the cGAS/STING pathway, which activates an innate immune response that can recruit tumor infiltrating lymphocytes (TILs), we postulated that misaligned chromosomes would be poorer inciters of immune activation than lagging or bridge chromosomes. Indeed, primary breast cancers with lagging and bridge chromosomes exhibit higher levels of TIL infiltration than cancers with equivalent levels of misaligned chromosomes. Together, these data indicate misaligned rather than lagging chromosomes represent the major mechanism of CIN in breast cancer and provide support for the immunostimulatory effects of specific types of CIN.

Endomembranes promote chromosome missegregation by ensheathing misaligned chromosomes

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Accurate chromosome segregation during cell division is essential for genomic stability. Errors in mitosis cause aneuploidy, which may be accompanied by micronuclei formation which is commonly associated with cancer. During mitosis, the mitotic spindle aligns chromosomes at the metaphase plate. However, if a chromosome becomes misaligned during metaphase and is not rescued, it can be missegregated: the daughter cells gain or lose a chromosome, causing aneuploidy. How chromosomes become missegregated during mitosis is not fully understood. In human cells at mitosis, the mitotic spindle is situated within an “exclusion zone” that is largely free of membranes and organelles. Outside of this zone is densely packed with endomembranes including the endoplasmic reticulum. Using live cell imaging and 3D EM, we found that misaligned chromosomes can become ensheathed in multiple layers of endomembranes outside of the exclusion zone. Semi-automated image analysis revealed that all ensheathed chromosomes (58/58) were outside the exclusion zone, at an average distance from the
metaphase plate of 1.44 times the pole-plate distance. While misaligned chromosomes that were not ensheathed were typically at the exclusion zone boundary (1.11 times the pole-plate distance, n = 57). By studying the fate of misaligned chromosomes in two different cell systems, we find that chromosome ensheathing promotes missegregation and micronuclei formation. Ensheathed chromosomes lack stable microtubule attachments and spindle assembly checkpoint (SAC) proteins are recruited to their kinetochores. Cells with ensheathed misaligned chromosomes are delayed in mitosis, but ultimately progress with missegregation suggesting that ensheathing partially interferes with SAC signalling. The micronuclei formed from ensheathed chromosomes have abnormal protein levels including nuclear protein complex components, which may contribute to genome rearrangement. In order to test whether chromosome ensheathing promotes missegregation, we developed a novel method to remove endomembranes in live mitotic cells. This process resulted in the rescue of ensheathed misaligned chromosomes to the metaphase plate. Together, our results reveal a novel mechanism which promotes chromosome missegregation: the ensheathing of misaligned chromosomes by endomembranes.

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Species-specific condensation failure at centromeres underlies subfertility in hybrid mice

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Reproductive isolation occurs when the genomes of two populations accumulated genetic incompatibilities that prevent inter-breeding. Incompatibilities in meiotic chromosome segregation processes could lead to hybrid infertility. However, it has not been directly tested if hybrid incompatibility in chromosome segregation machinery indeed serves as an evolutionary force to establish reproductive isolation by causing meiotic failure. Here, we tested this hypothesis using an interspecific mouse hybrid between Mus musculus and Mus spretus. Mus musculus x Mus spretus hybrid mice are viable but have fertility defects as their eggs suffer from extensive aneuploidy due to kinetochore-microtubule attachment defects in meiosis I (Sebestova J et al. 2012). To better understand the cell biological basis underlying this meiotic failure, we examined chromosome morphology in hybrid oocyte meiosis I. We found that hybrid oocytes have meiotic bivalents with stretched Mus musculus centromeres, while Mus spretus centromeres remained condensed properly. Our preliminary data suggest that this condensation defect is due to defective recruitment of condensin complex to Mus musculus pericentromeres. Further, the condensation defects were worse in early metaphase compared to late metaphase, which could affect the initial kinetochore-microtubule attachments. Consistent with this observation, less condensin complex localized on chromosomes in early metaphase. Consequently, Mus musculus centromeres lagged and mis-segregated in anaphase, explaining the formation of aneuploid eggs and hybrid subfertility. This study revealed chromosome condensation defects as a source of hybrid fertility defects.
Investigating the contribution of chromosome structure to micronuclei formation
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Micronuclei form when missegregated chromosomes recruit their own nuclear envelope and are a common marker of chromosome instability in cancer cells. Micronuclei also have unstable nuclear envelopes that frequently rupture, which has been linked to genome instability, upregulation of metastatic genes, and increased immune signaling. Preliminary data from our lab suggests that micronucleation of different chromosomes affects rupture frequency. Therefore, the identity of the micronucleated chromosome could significantly alter the downstream consequences of micronucleation. Previous work from our lab and others has shown that chromosome missegregation and micronucleation after mitotic disruption are non-random. However, there is a critical gap in our understanding of how chromosomal properties are responsible for these biases, and whether this bias differs between missegregation and micronucleation. We hypothesize that missegregation and micronucleation formation occur at separate frequencies, and that chromosome features connected to missegregation, including chromosome and centromere size, have additional effects on chromosome incorporation into micronuclei. To test this hypothesis we are optimizing a system to isolate micronuclei and sequence their contents. With this we will classify chromosomes enriched in micronuclei after different mitotic perturbations while also identifying chromosome features correlated with micronucleation. To determine how these features impact the likelihood of micronucleation, we are developing a system that adopts CLING, a dead CRISPR/Cas9 live imaging method, with sgRNAs targeting chromosome-specific repeat sequences to track individual chromosomes during mitosis. Together, these tools will allow us to comprehensively identify how individual chromosomes are impacted by mitotic errors and identify mechanisms that promote micronucleation. This will enable new insight into when micronucleus rupture is a likely driver of genome instability and innate immune responses in cancer tissues.

Mitotic Cells Can Elicit DNA Repair Synthesis in Response to DNA Damage Induced During Mitosis
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The ability of cells to repair damaged DNA is critical for the preservation of cell function. Defects in the ability of cells to repair damaged DNA can lead to developmental disorders and accelerated aging. Various details concerning DNA repair have been elucidated in interphase. Studies that have looked at mitotic DNA damage repair have found truncated responses. However, those studies relied on the treatment of cells with ionizing radiation or radiomimetic drugs which may lead to genome-wide alterations or off-target effects and thus possible differences in the way cells may respond to DNA damage. We investigated mitotic DNA damage responses by damaging chromosomes with a highly focused 780nm femtosecond laser. Our results show that the laser elicits complex DNA damage and that mitotic cells are capable of DNA repair synthesis. The synthesis was assessed by incubating the cells with a thymidine analog, 5-Ethynyl-2'-deoxyuridine (EdU). We found decreased EdU incorporation when cells
had compromised ATM and DNA-PKcs; increased DNA repair synthesis was observed when cells were incubated with PARP inhibitor. Furthermore, we found that cells could recruit downstream factors from the non-homologous end-joining pathway and that cells with compromised DNA-PKcs may recruit RAD51 during mitosis. Unlike previous studies, we observed recruitment of 53BP1 and BRCA1. In conclusion, studies on mitotic DNA damage responses may benefit greatly from laser-induced damage in that this damage may be restricted to one chromosome and allows for the production of high-density double-strand breaks in one region which could facilitate the visualization of DNA repair factors. Our results suggest that the mitotic DNA damage response may be more complex than previously thought.

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Cellular Adaptation To G2-phase Decatenation Checkpoint Is Controlled By MCPH1-CHK1-PLK1 Axis
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Catalytic inhibition of Topoisomerase II during G2 phase delays onset of mitosis due to the activation of the so-called decatenation checkpoint. This checkpoint is less known compared with the extensively studied G2 DNA damage checkpoint and is partially compromised in many tumor cells. We recently demonstrated that MCPH1, a gene mutated in primary microcephaly syndrome and potentially involved in tumorigenesis, is required for the adaptive response that triggers entry into mitosis despite Topo II catalytic inhibition. In order to better understand the molecular signals that contribute to bypass of this checkpoint, we have explored the interplay of MCPH1 with CHK1 and PLK1 within this pathway. Our results demonstrate that Chk1 function is required to sustain the G2 arrest induced by catalytic inhibition of Topo II. Interestingly, Chk1 loss of function restores adaptation in cells lacking MCPH1. Furthermore, we demonstrate that Plk1 function is required to bypass the decatenation checkpoint arrest in cells following Chk1 inhibition. Taken together our data suggest that MCPH1 is critical to allow checkpoint adaptation by counteracting Chk1-mediated inactivation of Plk1. Importantly, we also provide evidence that MCPH1 function is not required to allow recovery from this checkpoint, which lends support to the notion that checkpoint adaptation and recovery are different mechanisms distinguished in part by specific effectors. Our results add novel insight into the genetic requirements that provide cells with the capacity to bypass the G2 decatenation checkpoint. Furthermore, given the potential contribution of checkpoint adaptation to genome instability these data might be of importance to better understand recent evidences implicating MCPH1 in tumorigenesis.

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Binding of methylated H3 by Topoisomerase IIa is required for resolving tangled genomic DNA during mitosis for faithful sister chromatid disjunction
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Type II DNA Topoisomerase (TopoII) is an essential enzyme that decatenates tangled genomic DNA for sister chromatid disjunction through its unique Strand Passage Reaction (SPR) activity. Loss of the TopoII-SPR activity results in the formation of DNA bridges and/or Polo-like Kinase Interacting
CheckpointHelicase (PICH) coated ultra-fine bridges (UFBs) - indicators of flawed sister chromatids disjunction. Vertebrates carry two isoforms-Topolla and Topollb, known to share a conserved catalytic core. However, Topolla is indispensable during mitosis and its function cannot be compensated by Topollb. Topolla’s exclusive function in mitosis is attributed to it by its distinct C-terminal domain (CTD) that is dispensable for SPR. In this study, we demonstrated that the specific binding of Topolla to methylated histone governs the key difference between Topolla and Topollb function in mitosis. Topolla-CTD contains the Chromatin Tether (ChT) domain at the end of it that specifically interacts with methylated Histone H3. Using the Auxin Inducible Degron (AID) system in combination with the Tet-inducible expression of recombinant Topolla, we discovered that the ChT domain of Topolla plays a critical role in the complete resolution of PICH-coated UFBs during mitosis. Together with the in vitro chromatin binding assay using Topoll-CTD fragments, we identified critical residues that govern Topolla-specific function in binding to methylated H3 and resolving UFBs. Taken together, we propose that Topolla-CTD recognizes specific methylated H3-associated chromatin to facilitate the resolution of tangled genomic DNA during mitosis for proper sister chromatid disjunction.

Cilia and Flagella Assembly and Disassembly: Trafficking

P509

Intracellular mechanism for ciliogenesis requires Rab11 and Rab8 in vivo
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Kupffer’s Vesicle (KV), the zebrafish organ of asymmetry, which functions to establish the left-right body axes in a developing embryo. KV development commences as a migratory pool of cells (pre-rosette) which then establishes apicobasal polarity (rosette) and subsequently forms a ciliated fluid-filled space (lumen). The spatiotemporal dynamics of ciliogenesis during KV development have not been previously established. Our studies have shown that cilia begin to construct intracellularly (~100% of cilia present within the cells volume) prior to relocation to the plasma membrane where cilia mature and then the cilia protrude into the lumenal space. Previous in vitro cell culture studies have identified that the small GTPases, Rab8 and Rab11, are required for ciliogenesis where Rab11 recruits the Rab8 GTPase Exchange Factor (GEF), Rabin8, to activate Rab8 initiating cilia elongation. Our studies herein investigated if Rab11 and Rab8 work in a similar mechanism in vivo. Using a combination of live-cell imaging and optogenetic clustering to acutely disrupt Rab8 and Rab11 associated membrane function where we identified a unique role for Rab8 and Rab11. Rab11-membrane clustering does not block ciliogenesis per say but blocks the formed internal cilia from relocating and protruding into a KV lumen. KV lumens are also significantly smaller than controls when Rab11-membranes are optogenetically clustered. Whereas, Rab8 optogenetic clustering does not affect lumogenesis, but blocks cilia from constructing intracellularly. Our preliminary studies also suggest that Rab11 and Rab8 do not distribute to the same membrane compartments under non-clustering conditions during KV morphogenesis. These studies have identified a unique role for Rab11 and Rab8 during cilia formation in vivo, where Rab8-associated membranes are required for cilia’s initial formation and Rab11-associated membranes are required for proper lumogenesis and cilia protrusion into the luminal space.
Novel role of transcription factor NF-Y in regulating genes involved in cilia processes in early zebrafish development

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Dysregulation in cilia processes can result in a group of birth defects known as Ciliopathies. Cilia are outward projecting microtubule-based organelles, which can be classified broadly as motile or primary cilia. Several transcription factors (TFs) are known to regulate cilia processes, such as RFX and FOXJ1. However, these TFs do not explain how all ciliogenesis is regulated (primary cilia). Therefore, we hypothesize that other candidate TFs must regulate ciliogenesis, specifically that the TF nuclear factor Y (NF-Y) is acting in the early embryo to regulate cilia gene expression networks. NF-Y is a trimeric and ubiquitously expressed TF that has pioneer factor activity. To study NF-Y, we depleted it using a dominant negative construct and performed RNA-seq and ChiP-seq in zebrafish. I found that there were 347 directly regulated genes. GO- Term analysis of the directly regulated genes showed that NF-Y appears to regulate genes involved in cilia processes. Phenotypically, the deficient NF-Y fish had defects in eyes and head cartilage. To get a better understanding of the molecular mechanism, I determined the influence of NF-Y has on chromatin marks (H3K4me3, H3K27ac and H3K4me1) across time both genome-wide and specifically at cilia related genes. I found that in both conditions, NF-Y is acting as an enhancer. There was, however, slight differences in the chromatin patterns between the two groups, suggesting that NF-Y may be acting at cilia genes with a distinct molecular mechanism. To explain this unique molecular mechanism, I hypothesize that NF-Y is interacting with distinct protein partners, specifically RFX, which regulates ciliogenesis. Using bioinformatic analysis of ENCODE data of human cell lines, I found that there was substantial overlap of DNA binding sites between RFX and NF-Y subunits. This suggests that RFX and NF-Y may regulate cilia genes cooperatively. Overall, we found a novel role for the TF NF-Y in regulating cilia gene expression networks and found that NF-Y may be reorganizing chromatin and/or interacting with different TFs (RFX) to create a distinct molecular mechanism. Currently, we are measuring cilia characteristics and ciliogenesis in NF-Y depleted animals to determine if NF-Y deficiency leads to cilia defects. In addition, we are currently determining the extent of RFX and NF-Y interaction.

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Rab34 GTPase mediates ciliary membrane biogenesis in the intracellular ciliogenesis pathway

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Primary cilia form by two distinct pathways: an extracellular pathway in which the cilium grows out from the cell surface and an intracellular pathway in which the nascent cilium forms inside the cell. Membrane trafficking processes are critical in both cases, but the molecular and mechanistic differences between these modes of ciliogenesis are incompletely understood. Here we describe the role in intracellular ciliogenesis of Rab34, a Rab family GTPase required for ciliogenesis in RPE and NIH-3T3 cells. We show that Rab34 is required for formation of a ciliary vesicle at the mother centriole and
subsequent formation of the ciliary membrane. Analysis of Rab34 localization reveals that Rab34 is present specifically on assembling intracellular cilia, where it localizes to the ciliary sheath that later fuses with the plasma membrane. We further show that unusual divergent residues within Rab34’s GTPase domain are essential for its function and that GTP binding and turnover by Rab34 promote cilium assembly and maintenance. Lastly, given that Rab34 is found on assembly intermediates and membrane structures that are unique to the intracellular pathway, we tested whether Rab34 has a role in the extracellular ciliogenesis pathway employed by polarized MDCK cells. Notably, MDCK cells grown in 2D monolayers or 3D cysts assemble cilia independently of Rab34 and its close paralog Rab36. Together, our findings indicate that Rab34 is an atypical Rab GTPase that mediates ciliary membrane biogenesis specifically in the intracellular pathway of cilium assembly, thus revealing new context-specific molecular requirements for cilium function.

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**Basal body number scales with cell size in Tetrahymena thermophila**

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The control of basal body (BB) and ciliary number is important to ensure normal cilia-dependent fluid flow in multi-ciliated cells. In the ciliate, *Tetrahymena thermophila* BBs are organized into rows at the cell cortex and nucleate cilia for cellular motility. Importantly, the number of BBs remains relatively constant in *Tetrahymena* cells (Nanney, 1971). How this homeostatic balance of BB frequency is controlled during each cell cycle remains poorly understood. The mutant, *big1-1*, that causes larger cells with more BBs was used to investigate how cells regulate BB number (Frankel, 2008). *big1-1* mutant cells are longer and wider than wild type cells and possess supernumerary BBs. This suggests that BB assembly promiscuously increases in *big1-1* cells. To investigate how *big1-1* cells gain more BBs, we measured the rates of new BB duplication. Consistent with the increased number of BBs in *big1-1* cells, new BB duplication and assembly is elevated. Moreover, *big1-1* BBs mature more rapidly than wild type BBs. This suggests that rapid BB maturation renders new BBs competent to assemble more BBs, thereby increasing rate of new BB assembly at each cell cycle. Elevated temperature exacerbates the *big1-1* BB amplification and corresponding cell size increase. Conversely, media starvation of *big1-1* cells restores BB number and cell size to nearly normal levels. We find that the regulation of *Tetrahymena* BB number and cell size are coupled. The increase in cell size results from more BBs per ciliary row and an increase in the number of BB rows. Collectively, this suggests that *big1-1* mutant cells lengthen and widen to accommodate their supernumerary BBs and to maintain normal BB density. In summary, *Tetrahymena* BB number is tightly regulated thereby ensuring normal cell size. Frankel. (2008) *What do genic mutations tell us about the structural patterning of a complex single-celled organism?* Eukaryot Cell. 2008 Oct;7(10):1617-39. doi: 10.1128/EC.00161-08. Nanney, D.L. (1971), *The constancy of cortical units in tetrahymena with varying numbers of ciliary rows* J. Exp. Zool., 178: 177-181. doi:10.1002/jez.1401780204
Quantitative proteomic analysis of Chlamydomonas reinhardtii wild-type and long flagella mutant reveals differential expression
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Cilia and flagella are highly conserved organelles essential for normal health and development. The molecular mechanisms that regulate their assembly state, however, remain largely unknown. In Chlamydomonas, mutations in any of five long flagella (lf) genes result in cells that assemble flagella that are more than twice the length of wild-type cells. To learn more about the role of one of these genes, LF4, which encodes a MAP kinase, we used a phenotypic analysis along with a global proteomic approach to identify differentially expressed proteins in the flagella and cell bodies of lf4 and wild-type cells. Method: To examine cell body volume, cells were fixed with an equal volume of 1% glutaraldehyde and examined by phase contrast microscopy and the cell volumes were determined. Quantitative LC-MS/MS was performed on equal amounts of protein from purified cell bodies and flagella. Resulting data was analyzed using MaxQuant and Perseus software programs. Results: Microscopic analysis revealed a significant increase in size of lf4 cell bodies compared to wild-type cells. Analysis of the quantitative LC-MS/MS identified 1313 proteins from cell bodies and 692 proteins from flagella. We found that 11% of flagella proteins and 12% of cell body proteins were differentially expressed in lf4 and wild-type cells. Enrichment analysis revealed that proteins involved in protein synthesis, translation and ribosomal biogenesis were decreased in lf4 cell bodies. An increased expression was seen for proteins involved in energy production in lf4 cell bodies compared to wild-type. Analysis of the flagella proteome revealed increased expression for proteins involved in kinase and peptidase activity in lf4 while wild-type flagella had higher levels of expression for proteins associated with negative regulation of transcription and metabolism. Conclusion: The observation that lf4 cell bodies are larger than wild-type cells suggest that regulation of both flagellar length and cell body size are impaired. This could indicate that LF4 not only regulates flagellar length (or assembly state) but is also part of a signaling pathway controlling cell size and growth. The decreased expression for proteins involved in protein synthesis and ribosomal biogenesis in lf4 are surprising given the increased length of flagella and cell body size. The increase in protein levels for proteins involved in energy production could reflect an increased energy requirement necessary for flagellar motility with the lf4 flagella.

The Retromer Complex Regulates Centriolar Satellite Recruitment and Ciliogenesis
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Primary cilia are sensory organelles that coordinate a variety of signaling pathways, and defects in ciliogenesis result in a wide range of ciliopathies. Centriolar satellites are peripheral aggregates of centrosome/cilium protein complexes, and serve as key regulators of ciliogenesis, potentially by serving as reservoirs for centrosomal proteins. Early steps of ciliogenesis require ciliary vesicle formation accompanied by the removal of a distal end protein, CP110, from the mother centriole. This process is regulated by the endocytic regulatory protein Eps15 Homology Domain protein 1 (EHD1), and its interaction partner, Molecule Interacting with CasL-Like protein 1 (MICAL-L1). It remains unclear...
whether additional endocytic regulatory proteins are involved in ciliogenesis. In this study, we show that the retromer complex components, Vacuolar protein sorting-associated protein 26 (Vps26) and Vps35, as well as members of the BAR (Bin/Amphiphysin/Rvs) domain containing sorting nexin (SNX-BAR) protein family, SNX1 and SNX2, are required for ciliogenesis. In the absence of these proteins, CP110 fails to leave the mother centriole. Furthermore, we demonstrate an interaction between Vps26, Vps35 and CP110 by co-immunoprecipitation. Finally, we show that retromer depletion impairs the recruitment of centriolar satellites to the centrosome, potentially explaining the defective ciliogenesis. Taken together, our work reveals a novel role for the retromer complex in centriolar satellite recruitment and ciliogenesis.

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Aurora Kinase A proximity interactome reveals centriolar satellites as regulators of its function during primary cilium biogenesis

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Aurora kinase A (AURKA) is an evolutionarily conserved serine/threonine kinase that plays crucial roles in numerous cellular processes ranging from mitosis to primary cilium biogenesis. Although oncogenic mutations of AURKA is frequent in human cancers, its pleiotropic functions and complex spatiotemporal regulation has presented challenges in its therapeutic targeting. To overcome these challenges, it is essential to define the full range of its interactors and the mechanisms by which they regulate AURKA. Here, we applied the in vivo proximity-dependent biotin identification (BioID) approach and identified the first proximity interaction map of AURKA. The resulting interactome is composed of over 200 proteins involving multiple biological processes and cellular compartments. Importantly, it revealed previously undescribed interactions with centriolar satellites, which have emerged as important regulators of primary cilium biogenesis and functions. Using interaction and localization experiments, we identified AURKA as a new component of satellites and showed that satellites negatively regulate AURKA localization, activity and cellular abundance in quiescent cells. Increased AURKA activation at the basal body upon loss of satellites resulted in defective cilium assembly in serum-starved cells and enhanced cilium disassembly in serum-stimulated cells. Collectively, our results provide powerful resources for dissecting AURKA function and regulation, also uncover proteostatic regulation of AURKA by centriolar satellites as a new regulatory mechanism for its non-mitotic functions.

Cytoskeleton in Neuronal Morphogenesis

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Kpn1 coordinates cytoplasmic dynein complex in the anterograde migration

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KPNAs are known as the important nuclear transporting factors, and play essential roles in neurons such as memory, learning, and emotions by transmitting various molecules/information from cytoplasm to
nucleus, and from synapse to nucleus. KPNAs are multi-functional proteins and possess various roles depending on their subcellular localizations. However, function of KPNAs in the context of intracellular transport have not been well studied. Recently, it has been reported that functional abnormalities in KPNA1 are closely related to the development of neurological and psychiatric disorders such as schizophrenia and depression. We have conducted a comprehensive analysis of gene expression variations in KPNA1 knockout (KO) mice brains, and identified several characteristic genes that are vulnerable to drug treatment such as cytoplasmic dynein components. In this study, we examined the functions of KPNA1 in regulatory mechanisms of axonal trafficking and neuronal migrations, and tried to elucidate the role in neurological and psychiatric disorders by molecular level. We performed several imaging experiments with the dorsal root ganglion cells (DRGs) from 2-5 day old pups mice (C57BL/6J). First, dynamics of EGFP or mCherry conjugated KPNA1, KPNB1, dynein intermediate chain (DIC1) or neurospecific class III Tubulin (Tubb3) were examined by fluorescence recovery after photobleaching (FRAP) or dual-color imaging analysis. From those analysis, it has been suggested that KPNA1 were transported along axon, together with cytoplasmic dynein and Tubb3 in both anterograde and retrograde directions. Those results suggested KPNA1 have important roles in the signal transduction between synapse and nucleus. In addition, we tried biochemical analysis and obtained several promising results. Here we discuss new roles of KPNAs in intracellular transport on microtubules (MTs) and neuronal cell migration dependent on MTs traction.

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TRIMMING neurons: TRIM9 and TRIM67 regulate embryonic and adult neuronal morphogenesis
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TRIM9 and TRIM67 are neuronally-enriched E3 ubiquitin ligases that are essential for appropriate morphogenesis of murine embryonic cortical and hippocampal neurons as well as for appropriate responses to the extracellular guidance cue netrin-1. TRIM9 continues to be required for morphogenesis, migration, and synaptogenesis in adult-born neurons of the dentate gyrus. Deletion of either \textit{Trim9} or \textit{Trim67} results in subtle neuroanatomical defects, yet striking behavioral deficits, including spatial learning and memory deficits. These results indicate that TRIMs 9 and 67 are master regulators of form and function of developing embryonic and adult-born neurons, yet how they orchestrate this is unknown. Our previous work has identified a handful of actin polymerases, exocytic SNARE proteins, and netrin-1 receptors that interact with TRIM proteins. TRIM9 and TRIM67 regulate non-degradative ubiquitination of a subset of these proteins. We hypothesize TRIM9 and TRIM67 modulate morphogenesis by regulating membrane remodeling and cytoskeletal dynamics through additional cytoskeletal and membrane remodeling proteins, but these are currently unknown. To address this, we performed an unbiased proteomics study using the proximity-dependent biotin identification (BioID) approach. The BioID approach identified numerous candidate interaction partners. Gene ontology classification of these candidates indicated an enrichment of protein categories such as cytoskeletal regulators, cytosolic protein transporters, exocytosis and endocytosis regulators, and surprisingly also demonstrated enrichment of proteins necessary for synaptic regulation even though our experiments were performed prior to synapse formation. Additionally, cross-referencing the interaction candidates with the KEGG database also indicated an enrichment of proteins that play a role in Alzheimer’s disease. Using co-immunoprecipitation assays, we validated a subset of high priority
candidates, including Myo16, Coro1A, SNAP47, PRG-1, Tmod3, and Kinesin 3 family members KIF1A and B. For a subset of validated candidates, we utilized time-lapse TIRF microscopy to demonstrate dynamic colocalization with TRIM proteins at the cell periphery, including at the tips of lamellipodia and filopodia. Further analysis demonstrated the RNAi-based knockdown of the unconventional myosin, Myo16 in cortical neurons altered axonal branching patterns in a TRIM9 and netrin-1 dependent manner. Future studies will explore the mechanism leading to this phenotype. Analysis of other validated candidates will reveal how TRIM9 and TRIM67 function as master regulators of neuronal development and potentially identify novel targets and pathways disrupted in neurological disorders such as Alzheimer’s Disease.

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Myo16 and Coro1a function in TRIM-regulated neuronal morphogenesis
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To establish a functional and sophisticated neuron circuit, neurons progress through several developmental stages, including neurite initiation, neuron polarization, axon branching and guidance, and synaptogenesis. The dramatic neuronal shape change during these morphological events highly depends on the cytoskeleton remodeling machinery. Previously, we have discovered that two neuronally expresses E3 ubiquitin ligases, TRIM9 and TRIM67, regulate cytoskeletal dynamics particularly at filopodia during neuronal morphogenesis. We found that both TRIM9 and TRIM67 localize at the tips of the filopodia and regulate the ubiquitination of actin polymerase VASP, which in turn alters filopodia number and stability. In addition, neurons depleted with either TRIMs result in a loss of response to netrin-dependent axon branching. As E3 ligases usually interact with multiple substrates, we hypothesized that TRIM9 and TRIM67 may interact and regulate other cytoskeletal proteins during neuronal morphogenesis. Based on our recent proteomic experiments, we discovered that TRIM9 and TRIM67 have multiple candidate interacting cytoskeletal proteins. Here, we selected and validated two of the high-ranked candidates, myo16 and coro1A, and utilized both biochemical and neuron live-cell imaging assays to examine how TRIM9 and TRIM67 regulate these actin binding proteins during neuronal morphogenesis. We found that myo16 localizes at the tips of the filopodia, which is where the two ligases and VASP localize. Additionally, by utilizing immunoprecipitation assays, we demonstrated that myo16 interacts with TRIM9, TRIM67 and VASP. We also discovered that siRNA knockdown of myo16 in culture murine cortical neuron results in loss axon branching effect in the response of netrin. Ongoing work is examining Coro1A localization in murine cortical neurons and its role in neuronal developmental stages.

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KIFC1 steers the trajectory of migration of a neuron.
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Formation of the mammalian cortex involves migrating of neurons from the ventricular zone where they are born to their respective layers of the brain. Cytoplasmic dynein majorly known to generate pulling forces on microtubules which that allows the nucleus to move along the microtubule that are surrounding it. We have previously shown that populations of unattached microtubules exist in
migrating neurons and that regulation of sliding of these microtubules modulates the trajectory of neuronal migration. This can be thought of similarly to a herd of cattle moving from one place to another in which the linear trajectory of movement requires occasional midcourse corrections by a cattle-prod to prevent a combination of off-trajectory errors from taking the herd off course. Alternate sliding and non-sliding of centrosome-unattached microtubules in different locations of the migratory neuron can serve this function, as illustrated in our earlier work by neurons losing their trajectory when such sliding is experimentally obstructed. As for the nucleus, such an enormous structure cannot simply be tugged along the microtubules like a smaller organelle, but must be fluidly directed in the direction of migration. Here we investigated potential roles in neuronal migration of KIFC1, a minus-end-directed kinesin that is able to alternately slide and crosslink microtubules and also interact with membrane proteins. We propose the steering of the nucleus is majorly regulated by balance of sliding of microtubules by KIFC1 thereby regulating the trajectory of migratory neurons. Our studies indicate that KIFC1 indeed regulates the sliding and crosslinking of centrosome-unattached microtubules and enables the nucleus to rotate fluidly in the direction of neuronal migration. In both these ways, KIFC1 ensures the proper trajectory of the migrating neuron.

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Mechanism of 4-Nonylphenol Induced Neurodegeneration

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4-Nonylphenol (4-NP), an endocrine-disrupting compound (EDC), and a ubiquitous environmental toxin has been shown to affect brain development and may cause neurodegeneration, a condition that occurs in many neurological disorders, including Alzheimer’s (AD) and Parkinson’s (PD) diseases. 4-NP is produced in large quantities in the U.S. and used as raw materials for making detergents, pesticides, plastics, paints, cosmetics, and other industrial/household products that lead to its widespread release to the environment. However, the underlying molecular mechanism of 4-NP-induced neurodegeneration is not understood. **Goal:** The goal of this study is to elucidate the mechanism by which 4-NP causes neuronal disruption and induces neurodegeneration. **Methods:** PC12 cells (a model cell line to study neuronal structure and function) and neuronal cell SHSY5Y was used in this study. PC12 cells were differentiated to neuronal phenotype in the presence of NGF. Both NGF-differentiated PC12 cells and SHSY5Y cells were treated with 1, 5, and 10μM 4-NP and subjected to whole cell lysis, co-immunoprecipitation and confocal-scanning microscopy. In addition, proteomic analysis was performed with cytoskeletal fraction of PC12 cells (both control and NGF-differentiated) in the presence of 4-NP. **Results:** During neurodegeneration, the proper association and arrangement of cytoskeleton components are severely compromised. We found that 4-NP inhibits neurite formation, disrupts microtubule (MT) assembly and organization, and inhibits tubulin-Gβγ interactions in both in PC12 and SHSY5Y cells. Gβγ is an important component of the GPCR pathway, and its interaction with tubulin has been shown to be important for MT assembly and neurite outgrowth. We found that 4-NP interferes with expression/localization of microtubule-associated protein Tau. Tau is known to binds to MTs, and plays an important role in MT assembly and neurite outgrowth. During neurodegeneration, tau undergoes hyperphosphorylation and dissociates from MTs. We found that the interaction of tau with tubulin was decreased in the presence of 4-NP. High-Resolution proteomic analysis of a cytoskeletal fraction (CSKF) of PC12 cells reveals that 4-NP altered the proteomic landscape of the cytoskeleton (CSK) and increased the association of several proteins, including proteins of AD and PD pathways, with the
CSK. In addition, the PI3K/Akt/GSK3β pathway which is important for MT assembly/neurite outgrowth was also affected by 4-NP. **Conclusion:** These are important observations related to 4NP-mediated neuronal damages and provide a mechanism by which 4-NP induces a cascade of events leading to cytoskeletal disruption and neurodegeneration, and should provide essential information in assessing the environment risk of 4-NP.

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**Beta-iii-spectrin n-terminus is required for high-affinity actin binding and neuron function**

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Recent structural studies of β-III-spectrin and related cytoskeletal proteins revealed N-terminal sequences that directly bind actin. These N-terminal sequences are variable in length, composition and structure, and immediately precede a conserved actin-binding domain (ABD) composed of tandem calponin homology domains (CH1 and CH2). Here we investigated in Drosophila the significance of the β-spectrin N-terminus, and explored its functional interaction with a CH2-localized L253P mutation that underlies the neurodegenerative disease spinocerebellar ataxia type 5 (SCA5). We report that pan-neuronal expression of an N-terminally truncated β-spectrin fails to rescue lethality resulting from a β-spectrin null allele, indicating that the N-terminus is essential to β-spectrin function *in vivo*. Significantly, N-terminal truncation rescues neurotoxicity and defects in dendritic arborization induced by L253P. *In vitro* studies show that N-terminal truncation eliminates high-affinity actin binding caused by L253P, providing a mechanistic basis for rescue. Further, structure-based mutational studies identify residues in the N-terminus and CH domains that are critical in regulating actin binding. Our data support a model in which binding of the N-terminus promotes actin binding by both its direct interaction with actin and by facilitating the opening of the CH1-CH2 interface. Moreover, these data suggest that N-terminal sequences may be useful targets for modulating the aberrant actin binding associated with SCA5 and diseases caused by spectrin-related proteins.

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**Cannabinoid Receptor 1 regulates growth cone filopodia and optic axonal projections in the optic tract of Xenopus laevis tadpoles**

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Previous studies show that the main cannabinoid receptor in the brain-CB1R- is required for normal growth cone filopodia and axonal projections in a variety of developing neurons. However, questions still remain about exactly how CB1R functions to modulate developing neuronal circuits. Here we applied pharmacological reagents to determine how CB1R influences growth cones filopodia and axonal projections in the optic tract of whole brains from Xenopus laevis tadpoles. Our data show that GFP expressing optic axons exposed to a CB1R inverse agonist had larger growth cones with significantly more filopodia than control growth cones. In contrast, growth cones of optic axons exposed to a CB1R agonist were smaller and had fewer filopodia than control growth cones in situ. However, both the CB1R inverse agonist and agonist resulted in optic axons that were overly dispersed and followed more wavy trajectories in the optic tract of whole brains. These data suggest that inactivation and activation of
CB1R increase and decrease number of growth cone filopodia, both of which lead to increased defasciulation and undulation in optic axons in situ. More broadly, our results imply that an intermediate level of CB1R regulated growth cone filopodia are required for normal fasciculation and pathfinding of optic axons in the tract of Xenopus laevis tadpoles.

P523

Vasp ubiquitination regulates actin dynamics and neuronal morphology

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In neurons, filopodia are critical for neuritogenesis, axon guidance, and dendritic spine formation. Defects in these processes can result in improper synaptic connectivity, neurodevelopmental disorders, and psychiatric syndromes. The actin polymerase VASP localizes to the filopodial tip complex and influence actin dynamics. Previously, the Gupton lab showed VASP transiently co-localizes with the E3 ubiquitin ligase TRIM9 at the filopodial tip. TRIM9 was required for the reversible, non-degradative ubiquitination of VASP and this modification was associated with decreases in growth cone filopodia number and stability. Although the dynamic actin cytoskeleton and VASP are also appreciated to play important roles in the postsynapse, it is not known how VASP activity is regulated in dendritic filopodia and the maturing dendritic spine. Here we show VASP, TRIM9 and ubiquitinated VASP (VASP-Ub) localize to the PSD following differential centrifugation, suggesting a role for VASP-Ub in dendritic spines. Cultured murine cortical neurons overexpressing VASP or VASP-KR (a non-ubiquitinable construct) exhibit no significant changes in dendritic filopodia number. Although the guidance cue netrin promotes synaptogenesis, neurons overexpressing VASP or VASP-KR demonstrated a decrease in dendritic filopodia number following netrin treatment. To understand this puzzling result, we are currently utilizing live cell imaging to quantify the lifetime of these filopodia. Ongoing work is also examining Trim9 deletion, as well as VASP and VASP-KR overexpression, on dendritic spine number, maturity and synaptic plasticity. Future work will explore the mechanistic impact of ubiquitination on actin-VASP interactions through in vitro biochemical reconstitution assays.

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The chaperonin CCT is required for cell-type specific dendritic diversity via regulatory effects on the microtubule cytoskeleton

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Cellular proteostasis is indispensable to the survival and function of all cells. Distinct from other cell types, neurons are long-lived, exhibiting architecturally complex and diverse multipolar morphologies that can span great distances. These properties present unique demands on proteostatic machinery to dynamically regulate the neuronal proteome in both space and time. Molecular chaperones play critical roles in proteostasis, however little is known regarding their functional requirements in dendrite development. Chaperonin-containing tailless complex polypeptide 1 (CCT) is a hetero-octameric, ATP-dependent chaperonin that has been implicated in folding of approximately 10% of the cellular proteome, including the two major cytoskeletal proteins, actin and tubulin. Although CCT has been studied in axons, especially in conjunction with Hereditary Spastic Paraplegia, a genetic disease marked
by advancing neuropathy, the potential function of CCT is not well understood in dendrites. Therefore, we have investigated CCT chaperonin function in the dendrites of *Drosophila melanogaster* multidendritic (md) sensory neurons of the peripheral nervous system using neurogenetics; *in vivo* imaging; and neuromorphometric quantitative analyses. Loss-of-function genetic analyses reveal that disruption of each of the eight CCT subunits results in dendritic hypotrophy in dendritically complex Class IV (CIV) polymodal nociceptive sensory neurons whereas no dendritic arbor changes were observed in the architecturally simpler Class I (CI) or II neurons. Furthermore, combined mutation of two CCT subunits produced synergistic phenotypic defects in dendritic development. Consistent with phenotypic findings, our transcriptomic analyses of these sensory neuron subtypes reveal that CCT is most highly expressed in CIV neurons and shows the lowest levels of expression in CI neurons. Together, these findings suggest that different neuronal types have differential reliance on CCT function. Dendritic arborization is highly dependent on the organization, maintenance and dynamic modulation of the cytoskeleton to not only regulate cell shape, but to also facilitate trafficking of vesicular cargo and organelles to support growth, development and function. Intriguingly, we find that CCT subunit mutation in CIV neurons results in a dramatic reduction in the levels of stable microtubules, however F-actin levels remained stable. These findings suggest that CCT may mechanistically regulate dendritic architecture via cell-type specific effects on proper folding of tubulin. Collectively, our investigations have revealed regulatory roles for CCT in directing cell-type specific dendritic morphogenesis which may be linked to the role of CCT in the proper folding of tubulin.

**Digestive and Excretory Organs**

**P525**

*Increased apoptosis in differentiated renal proximal tubules derived from ctns hiPSCs*

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The objective is to determine, via studies with renal proximal tubule (RPT) cells derived from cystinotic induced pluripotent stem cells (hiPSCs), whether the renal Fanconi Syndrome in nephropathic cystinosis can be explained by oxidative stress-induced apoptosis. Cystinosis results from autosomal recessive mutations in the *ctns* gene, encoding for cystinosin, a transport protein responsible for extrusion of cystine from lysosomes. When cystinosin is defective, lysosomal cystine levels increase to such an extent that cystine crystals form. In infantile cystinosis, kidney function in particular becomes impaired several months after birth due to reduced solute reabsorption by the RPT. Infants with this disorder to exhibit the Fanconi Syndrome (increased urination, thirst, dehydration and acidosis), impaired growth, rickets, and ultimately, a limited lifetime. In order to develop new avenues of therapy, hiPSCs have been developed from cystinotic fibroblasts with a 57 kb deletion in *ctns* (as well as normal counterparts). The cultures were transduced with 3 vectors (pCXLE-hUL encoding for L-myc and Lin28; pCXLE-hSK encoding for Sox2 and Klf4, and CXLE encoding for Oct3/4). The transforming oncogene EBNA was no longer observed after 3 months. Nevertheless, the CTNS and wildtype hiPSCs retain pluripotency, as indicated by the formation of embryoid bodies with all 3 germ layers. Both WT and CTNS hiPSCs have been differentiated into RPTs following the method of Lam et al. (JASN 25:1211 (2014)). Tubule structures are observed with RPT specific markers, including apical villin, dipeptidyl peptidase 4, NHE3, and NPT2a. The Fanconi Syndrome can be elicited in normal children following treatment with ifosfamide, a prodrug, whose metabolite chloracetaldehyde (CAA) affects the RPT. CAA (50 µM) induced apoptosis in RPTs
derived from the hiPSCs, the frequency of apoptosis being 4 times higher in RPTs derived from CTNS hiPSCs. Similarly, apoptosis observed following hypoxia (1% O₂, 36 hrs) was 3 times more severe in RPTs derived from CTNS vs. WT hiPSCs. The increased apoptosis may be due to defective autophagy due to the lysosomal defect. The results indicate that CTNS RPTs derived from hiPSCs may be used as an effect tool to identify the underlying mechanisms resulting in altered renal function in this disease, and the basis for developing new therapies.

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**ZIP14 and the regulation of systemic manganese metabolism**

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Manganese is essential for human health. As a newly identified manganese importer, ZIP14 is abundantly expressed in the liver and small intestine, the two major organs involved in the control of manganese metabolism. Patients with loss-of-function mutations in ZIP14 developed severe neurological disorder due to manganese hyper-accumulation in the brain; similarly, mice with whole-body Zip14 knockout displayed manganese loading in the blood and brain, indicating an indispensable role for ZIP14 in maintaining systemic Mn homeostasis. To examine the functions of ZIP14 in the liver and intestine, we generated tissue-specific Zip14 knockout mice. Liver specific Zip14 knockout mice did not develop systemic manganese overload. However, intestine-specific inactivation of Zip14 increased body manganese load in mice, suggesting that intestinal ZIP14 plays an important role in controlling the whole body manganese homeostasis. Our results provide important insight into the disease mechanism underlying manganese hyperaccumulation observed in individuals lacking functional ZIP14.

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**Three-dimensional organoid cell culture models for the study of liver injury**

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**Background and Aim:** Liver injury usually occurs as a result of immune-mediated or direct injury to the hepatocytes. Hepatocytes are the first cells to process dietary contents after absorption and can be exposed to injuries from ingested toxins, alcohol, drugs, and infectious pathogens. During injury, hepatocytes engage in cross-talk with multiple cellular subsets including adipocytes, endothelial cells, Hepatic Stellate Cells (HSCs), and infiltrating immune cells, resulting in the release of Reactive Oxygen Species, proinflammatory signals, proliferation-associated cytokines, and the activation of repair pathways. This broadly canonical injury response then leads to fibrosis and cirrhosis which occurs irrespective of etiology. Representative in vitro modeling of liver injury is essential for developing anti-fibrosis drugs for patients at risk of developing liver fibrosis and cirrhosis. A 3D Organoid cell culture model is a collection of cells with several cell types, that develop from stem cells or organ progenitors which self-organise through cell sorting and spatially restricted lineage commitment; similar to organogenesis in vivo. This self-renewing primary cell culture has tissue-specific architecture, intercellular heterogeneity and cell-cell signaling. We aimed to model liver injury in vitro for the purposes of assessing pre-clinical anti-fibrosis drugs. **Methods:** We modelled the molecular biology of liver injury in vitro using mouse liver organoid cell cultures in a trans-well system with JS1 (mHSC) cell
These cells were activated either by TGF-β, TNF or hypoxia induced apoptotic bodies. We also tested the effects anti-fibrotic drug Halofuginone using our model. We measured changes in gene expression using custom fibrosis microarray chips, protein expression of SMA and Collagen I with immunofluorescent labelling and cell ultrastructure with electron microscopy. **Results:** We were able to observe changes in fibrosis related gene expression of our liver organoids and HSCs in vitro. These were also a significant decrease in HSC viability caused Halofuginone treatment. HSCs were activated in the injury model and produced Collagen Type I in response to injury. There were also significant changes in organoid ultrastructure caused by exposure to injury signals from HSCs, TNF treatment and hypoxia. **Conclusion:** Organoids may be useful to model liver injury and anti-fibrosis drug interventions in vitro, but further in-vitro experiments on organoids are required to delineate their role as an ex-vivo model of injury and utility as a means to assess future anti-fibrotic therapies.

**P528**

**Measurement of collagen metabolism and metalloprotease production demonstrates that primary human gingival fibroblasts retain inflamed tissue profile in serum free culture**

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Periodontitis is a common but easily preventable disease that affects many adults. It is characterized by a state of chronic inflammation that attacks the host’s gum tissue and periodontal ligaments. If severe and left untreated, periodontitis can cause tooth decay and tooth loss. Smoking is a significant risk factor because it causes an imbalance of the normal collagen type 1 metabolism that occurs via metalloproteinase actions. In particular, matrix metalloproteinase 8 (MMP-8) is a key player in this collagen degradation. In this study, primary human gingival fibroblasts (HGF) isolated from patients who were identified as inflamed non-smoker (IFN) and inflamed smoker (IFC) were cultured in serum-free media over a 72 hour period. Samples from n=6 patients reflected 83% African American, 67% female and 33% self-reported smokers. We measured production of MMP-8, intact and degraded collagen type I in the secreted fraction (conditioned media) and in the incorporated extracellular matrix (ECM). In the secreted and incorporated fractions the 24hr time point was optimal for measuring collagen metabolism as detectable levels approached zero by 72 hours. HGF isolated from smoker patients (IFC) produced more degraded collagen (400% more secreted and 25% more incorporated) than HGF isolated from the non-smoker (IFN). In addition, MMP-8 was secreted by IFN and IFC fibroblasts in the range of 0.05ng/culture and incorporated in the range of 0.076ng/culture (IFN) and 0.014ng/culture (IFC). MMP13 was undetectable in both the secreted and incorporated fractions. This data supports the hypothesis that inflamed tissue isolated from a current smoker exhibits a profile of collagen metabolism in line with more acute diseased state (fibrotic collagen profile). In addition, the culture of primary human gingival fibroblasts in serum-free medium for 24 hours is a viable method to study both the disease mechanism of periodontitis and potential therapeutics to treat the disease.
Examining the relationship between brain-specific angiogenesis inhibitor - 1 (BAI1) expressing myo/nog cells and hepatic stellate cells in liver injury

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Myo/Nog cells, which express the skeletal muscle specific transcription factor MyoD, and the bone morphogenic protein (BMP) inhibitor noggin, are also characterized by specific immunohistochemical labeling with the G8 monoclonal antibody (mAb) whose epitope was recently identified as brain-specific angiogenesis inhibitor 1 (BAI-1). These cells are important regulators of normal development, progenitors of myofibroblasts in the ocular lens, neuroprotective in the retina, and respond to wounding in multiple tissues. However, the role of Myo/Nog cells modulating tissue injury and fibrosis in the liver has not been studied. We used an immunohistochemical approach to examine the response of Myo/Nog cells to acetaminophen induced acute liver toxicity, as well as their relationship to human hepatic stellate cells known to be involved in the liver injury response. Liver tissue was obtained from mice following acetaminophen overdose over a time course of 96 hours. LX-2 human hepatic stellate cells were used to study the effects of mechanical injury (scratch assay) on expression of BAI1 using the G8 mAb. Immunofluorescent analysis using anti-BAI1, anti-noggin, and anti-glial fibrillary acid protein (GFAP), a stellate cell marker, showed that there is a resident population of Myo/Nog cells in the liver that decline in number during the first 24 hours after acetaminophen overdose and then increase, particularly in the region near the central vein (zone 3) where hepatocyte necrosis is greatest. These cells peak in number 48 hours after acetaminophen administration, the time of maximal liver injury as previously documented by serum biomarkers. Interestingly, a subpopulation of LX-2 human hepatic stellate cells were labeled with the anti-BAI1 mAb, and the proportion of cells that express this Myo/Nog cell marker increases after wounding, peaking at 24 hours. These studies suggest that Myo/Nog cells respond to acute liver injury by increasing in number and/or migrating toward the site of injury. Whether these cells play a role in mediating progression of liver injury or promote recovery via liver regeneration needs to be further evaluated.

Assessing the effects of a novel metalloprotease inhibitor, Extracellular Matrix Protection Factor 2, on primary human gingival fibroblast’s metabolic viability as determined by total protein production

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In the pathogenesis of periodontal disease, collagen, produced by gingival fibroblasts, is degraded by upregulated metalloproteases (MMPs) leading to the loss of structural integrity of the extracellular matrix. In this study, we investigated the effects of a novel MMP inhibitor, Extracellular Matrix Protection Factor-2 (ECPF-2), on human gingival fibroblast’s (HGVF) metabolic activity. ECPF-2 reversibly inhibits MMP-8 by blocking its interaction with collagen type I, but the effects of this novel therapeutic on HGVF behavior in culture is unknown. Cells were enzymatically released from samples harvested
during oral surgery and expanded to passage 2. Subconfluent cultures were transferred to 0.1% fetal bovine serum containing DMEM media overnight, transferred to serum-free medium (SFM) and reared for 72 hours. Based on total protein, total RNA and collagen type I production, 24 hours in serum-free media alone was optimal. Passage 2 cultures were then treated with 5ug ECPF-2, 50ug ECPF-2, or control serum-free media. After 24 hours treatment with ECPF-2, conditioned media was collected and the cell layer was extracted with 0.5% CHAPS buffer. We measured the total protein production in cultures isolated from normal non-inflamed (NTN); inflamed non-smoker (IFN); inflamed previous smoker (IFP) and inflamed current smoker (IFC) patient samples using the Pierce Modified Lowry Protein Assay. Using this methodology, we found that regardless of patient pathology or treatment conditions, all cultures contained approximately 1.3-1.5ug/ml/culture of total protein. There was no statistically significant difference in measured total protein between the treated or control samples. Based on these findings, we suggest that the defined culture system allows for viable metabolism in serum-free medium when treated with the novel MMP inhibitor, ECPF-2. This given, the culture system can be used to investigate the potential therapeutic effects of ECPF-2 on human gingival fibroblasts isolated from periodontal diseased tissue.

P531

**Endosomal-related function of rab21 in enterocytes participates to intestinal homeostasis via egfr pathway regulation**

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Membrane trafficking characterizes the vesicular transport of proteins and macromolecules throughout the cell. Impacting trafficking affects signalling events and conducts to dramatic consequences on cell homeostasis. Enterocytes (ECs) are differentiated cells that constitute about 80 % of the intestinal epithelium. Given their absorptive function, they display a high membrane trafficking flux. Notably, defects in endocytic pathways can affect ECs function and lead to intestinal bowel diseases. However, how does trafficking regulate intestinal tissue homeostasis is poorly understood. Rab21 is a small GTPase involved in early endosomal trafficking. It was first identified in human intestinal cells and is expressed in ECs. Importantly, Rab21 levels are drastically decreased in ECs upon intestinal imbalance. Using the Drosophila intestine as an in vivo model system, we investigated EC-specific functions for Rab21 in gut homeostasis. We monitored and assessed Rab21 loss of function in adult ECs via the use of a cell specific temporal inducible system. Rab21 silenced in ECs conducted to multiple defects. As such, Rab21 depleted guts showed severe intestinal morphology abnormalities. Furthermore, normal homeostasis was deregulated, and a gain in mitotic cells, associated with increased cell death was observed. Interestingly, we found that the IL-6 like cytokine, Upd3, was significantly induced, as well as the activity of the JAK/STAT pathway. Using a RNAi screen, we identified autophagy, specific early endosomal membrane trafficking regulators and Egfr-related genes phenocopying the Rab21-induced hyperplasia and inflammation. Coherent with these results, we observed that Egfr signalling was upregulated in fly intestines depleted for Rab21 and that overexpression of a dominant negative form of Egfr upon Rab21 depletion was sufficient to rescue Rab21-related phenotypes. To further characterize Rab21 function on Egfr regulation in ECs, we defined Rab21 EC interactome by in vivo proximity labeling. Interestingly, we found Stam, a negative Egfr regulator, as being an interactor for Rab21. Altogether, our data indicate that Rab21 plays an important role in EC-mediated intestinal homeostasis through Egfr
signalling regulation. Although its specific cellular function on Egfr regulation remains to be defined, we show that Rab21 protects EC against cell death by downregulating Egfr signalling.

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Fibrosis in experimental nonalcoholic steatohepatitis in mice is attenuated by extracellular vesicles from hepatocytes

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Background: Non-alcoholic fatty liver disease (NAFLD) describes a spectrum of diseases ranging from simple fatty liver (steatosis) to non-alcoholic steatohepatitis (NASH) and cirrhosis which are characterized by fibrosis and decreased liver function. NASH is initiated by multiple hits to the liver (insulin resistance, obesity, diabetes, gut endotoxin, hyperlipidemia) causing lipotoxicity-induced oxidative or ER stress in hepatocytes, infiltration of inflammatory cells, and activation of fibrosis-producing hepatic stellate cells (HSC). In this study we investigated whether extracellular vesicles (EVs) from human hepatocytes are therapeutic for experimental NASH in mice. Methods: 7-week C57BI/6J mice were fed 60% fat choline-deficient amino acid-defined (CDAA) diet ad libitum for 4, 8 or 12 weeks. Some mice also received 1-3 weekly i.p. injections of 4 x 10^8 or 2 x 10^9 hepatocyte EVs that had been collected by ultracentrifugation of 48-hr serum-free conditioned medium from human HepG2 cells. Mouse livers were evaluated for NASH-like pathology by histology and were stained for the presence of collagen, αSMA or key inflammatory cell types. Expression of fibrotic, inflammatory or lipid metabolism genes was determined by RT-PCR. Serum chemistries were performed for key markers of liver function or NASH. Results: Mice fed high-fat CDAA diet for 4-12 weeks exhibited increased serum alanine aminotransferase levels, decreased serum cholesterol or triglyceride levels and extensive lipid droplet accumulation (steatosis), inflammation, αSMA production, and progressive fibrosis in the liver. EV administration did not restore serum chemistries or reduce steatosis but caused attenuation of inflammation, αSMA production, collagen deposition, and expression of genes related to inflammation (e.g. CCL3, CCL5), fibrosis (e.g. COL1A1, CCN2, TIMP1) or lipid metabolism (e.g. FASN, SREBP1c). At the 12-week time point, effects on gene expression were most pronounced at the highest EV dose and frequency of administration. Conclusion: In experimental NASH, EVs from hepatocytes are anti-inflammatory and anti-fibrotic in the liver and suppress hepatic lipid-, inflammation- and fibrosis-related gene expression. Supported by R21AA025874 and R01AA027502 awarded to DRB.

Dynamics and Regulation of Nucleocytoplasmic Transport

P533

Drosophila Wash and the Wash regulatory complex function in nuclear envelope budding

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Nuclear envelope (NE) budding is a phenomenon wherein large macromolecular complexes, which are too large to be exported through nuclear pores, are packaged and expelled through the nuclear membranes. This pathway shares many similarities with the nuclear egress of herpesviruses, suggesting
NE budding may be an endogenous host pathway utilized by the virus. Although a general outline of the cellular events occurring during endogenous NE budding can be inferred from the proteins known to be involved and visualization of the process, very little is yet known about the molecular machinery and mechanisms underlying the physical aspects of NE bud formation. Using genetics, biochemistry, and super-resolution imaging, we identify Wash, the Wash regulatory complex (SHRC), capping protein, and Arp2/3 as novel molecular components involved in the physical aspects of NE bud formation in a Drosophila model system. Interestingly, depletion of WASH in salivary gland nuclei causes wrinkled nuclei and loss of nuclear buds, whereas knockdown of SHRC results only in the loss of nuclear buds. Using double immunofluorescent staining and point mutations we show that Wash affects NE budding in two ways: 1) indirectly through general nuclear lamina disruption via an SHRC-independent interaction with Lamin B leading to inefficient NE bud formation, and 2) directly by blocking NE bud formation along with its SHRC. We also show that Wash requires Arp2/3 and capping protein for NE bud formation, suggesting Wash’s ability to form new branched actin networks may be needed. By mass spec and native PAGE, we show that Wash acts as part of multiple, separable nuclear complexes to affect its diverse set of nuclear properties/events. We are currently investigating the specific function of the protein components of each of these complexes to further elucidate Wash’s mechanistic role in NE budding and other nuclear processes. NE budding is emerging as an important endogenous nuclear process, as well as sharing many similarities with herpesvirus nuclear egress mechanisms, opening up potential avenues for exploration in both normal and disease biology.

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Differential regulation of mRNA transport during acute stress through phase separation of the nuclear mRNA export factor Nab2

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Unidirectional transport of mRNA from the nucleus to the cytoplasm via nuclear pore complexes is an essential step in eukaryotic gene expression. Although factors involved in mRNA transport have been characterized, a comprehensive, mechanistic understanding of this critical process and its regulation is lacking. Here, we combine state-of-the-art real-time single RNA imaging and an inducible degron system to demonstrate that acute depletion of the budding yeast DEAD-box ATPase Dbp5 causes rapid nuclear accumulation of single labeled mRNAs in vivo and dramatic changes in nuclear dynamics of RNA export factors. In particular, the essential export factor Nab2 ceases to shuttle between the nucleus and cytoplasm and forms a gel-like structure throughout the nucleus. This potential phase-separation phenotype can be recapitulated in vitro, with Nab2 forming RNA-dependent liquid droplets, which dissolve in the presence of Dbp5. Intriguingly, nuclear Nab2 condensation also occurs in physiologically relevant stress conditions, where bulk mRNAs are globally retained in the nucleus. Importantly, stress-induced mRNAs can overcome nuclear retainment to elicit a timely cellular stress response, suggesting that cells use selective retention in or release from nuclear condensates to re-wire mRNA export during stress. These findings establish a novel layer of gene expression regulation and provide key mechanistic insights into mRNA transport.
**P535**

**Nuclear transport is regulated by cytoskeletal forces transmitted via the LINC complex**

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The LINC (Linker of Nucleoskeleton and Cytoskeleton) complex spans the nuclear envelope to mechanically couple the nucleus to the cytoskeleton. The outer nuclear membrane nesprins connect the nucleus to actin, microtubules and cytoplasmic intermediate filaments to help mechanically integrate the nucleus within the cell. The inner nuclear membrane SUN domain proteins interact, directly or indirectly, with nuclear lamins, various inner nuclear membrane proteins and chromatin. Utilizing a light-inducible reporter of nuclear transport (LINuS) we have serendipitously identified that modulation of LINC complex constituents leads to altered nucleocytoplasmic exchange. Effectively disrupting the LINC complex by co-depletion of both SUN domain proteins, Sun1 and Sun2, leads to an increase in nuclear transport. Perturbation of the LINC complex by transient expression of GFP-KASH4, which outcompetes endogenous nesprins for Sun1 and Sun2 binding, leads to a similar increase in nuclear transport. Depletion of Sun2, alone or in combination with A-type lamins led to a similar increase in nuclear transport as perturbation of the entire LINC complex. In contrast, depletion of Sun1 did not alter nuclear transport, unless it was co-depleted with A-type lamins, in which case it led to a decrease in nuclear transport. In Sun2 depleted cells we observed a change in the distribution of GFP-Ran, a key regulator of nuclear transport, with marked accumulation at the nuclear envelope. Finally, we observed that pharmacological depolymerization of actin or microtubules caused an increase in nuclear transport. Collectively, these data suggest that decreasing nuclear forces through either the disruption of the LINC complex, depletion of Sun2, or the depolymerization of actin and microtubules leads to an increase in nuclear transport. Ongoing studies are evaluating the role of cell shape, cellular tension and nuclear mechanics in the regulation of nuclear transport and the underlying mechanisms.

**P536**

**Importin alpha2 regulates cytoplasmic histone dynamics in Drosophila**

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The rapid nuclear divisions in early embryos demand a continuous histone supply, yet histone delivery to the nucleus needs to be tightly regulated to prevent overaccumulation-induced damage. In Drosophila embryos, regulation of the histone variant H2Av is mediated by its sequestration to lipid droplets (LDs), cytoplasmic fat storage organelles. H2Av is transiently bound to the LD-anchoring protein Jabba and continuously exchanges between LDs, thus providing a measured, yet steady pool of cytoplasmic H2Av for nuclear import. H2Av exchange is developmentally regulated, stopping abruptly at the mid-blastula transition (MBT). Using proteomics and immunostaining, we now find that Importin-α2 (Imp-α2) is dramatically enriched on LDs just as H2Av exchange ceases, i.e., Imp-α2 levels on LDs increase substantially in post-MBT embryos, consistent with the possibility that Imp-α2 negatively regulates H2Av exchange. Embryos from Imp-α2 mutant mothers do not develop, preventing a test of H2Av dynamics at the MBT. Using Fluorescence Recovery After Photobleaching (FRAP), we find that H2Av already exchanges between LDs during oogenesis and that in Imp-α2 mutants this exchange is...
abolished. The latter result suggests that Imp-α2 positively regulates H2Av dynamics. Enrichment of Imp-α2 on LDs is Jabba dependent, and luciferase complementation reveals that Jabba can physically interact with Imp-α2. Our working model is that Imp-α2 modulates Jabba’s ability to bind H2Av, and release of H2Av and Imp-α2 from Jabba are coupled. This model explains both why Imp-α2 is required for H2Av exchange and why its LD accumulation leads to static H2Av on LDs. Deletion analysis identified three distinct regions in Jabba that mediate LD targeting, H2Av binding, and Imp-α2 interaction, respectively. In particular, we have mapped a 4-amino-acid motif specifically required for Imp-α2 interaction. We are now using Jabba mutants lacking this motif to test whether Imp-α2-Jabba interactions are necessary to promote H2Av exchange between LDs and/or to stop exchange post-MBT. Finally, we have discovered that Imp-α2 is dephosphorylated post-MBT, a change that requires neither Jabba binding nor localization to LDs. Using point mutants in a key phosphorylation site, we will test whether it is the change in Imp-α2’s phosphorylation state that drives recruitment to LDs or halts H2Av exchange.

P537

5-fluorouracil and gemcitabine alter nuclear transport in panc1 cells

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Ran is a major regulator of nuclear transport, and disruption of its localization can lead to apoptosis. The chemotherapy 5-Fluorouracil (5FU) increases nuclear pore permeability causing the Ran concentration to be higher in the cytoplasm than inside the nucleus, altering nuclear transport and causing cell death. We have shown that combination treatments of 5FU with gemcitabine further increase cytoplasmic Ran in HeLa cells, and lead to cell death by inducing accumulation of p53 and p27 in the nucleus. Because gemcitabine resistance often occurs in pancreatic cancer, we sought to further study the effects of this combination in PANC1 pancreatic cancer cells to determine if the addition of 5FU can help overcome resistance. Our results showed that 5FU increased Ran localization to the nucleus, while the gemcitabine and combination treatments decreased nuclear Ran localization from the control but were not different from each other. Immunoblotting showed that Ran expression increased in all treatments, indicating that Ran is being mislocalized to the cytoplasm to a higher degree; as we see a decrease in nuclear Ran despite higher protein levels and increases in overall Ran in the gemcitabine and combination treatments. Additionally, the slight increase in nuclear Ran seen in the 5FU treatment may be due to increased nuclear Ran rather than a lesser ability to disrupt the Ran gradient. This outcome implies that 5FU and gemcitabine together may not work better at disrupting Ran localization than gemcitabine by itself as the amount of nuclear Ran between the two treatments is not significantly different, emphasizing the importance of testing particular combinations of chemotherapies in specific cell types to identify variations in response. Immunofluorescence and immunoblotting experiments of tumor suppressors p53 and p27 to determine how the treatments effect nuclear retention of these proteins are currently underway as well as MTT assays to assess how viability is affected by these treatments. These additional experiments will help determine the overall impact of the combination of 5FU and gemcitabine in PANC1 cells. This study can shed light on the mechanisms utilized by 5FU, gemcitabine, and a combination of the two drugs to induce apoptosis in pancreatic cells, and can provide insight on ways to circumvent gemcitabine resistance.
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Nucleocytoplasmic transport of intrinsically disordered proteins studied by high-speed super-resolution microscopy
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The majority of cancer-related proteins, as well as many proteins associated with neurodegeneration, diabetes, and cardiovascular disease are by intrinsically disordered proteins (IDPs), or contain large regions of disorder. We found a new mechanism of nucleocytoplasmic transport exhibited by IDPs while transiting through the nuclear pore complex (NPC). By using high-speed super-resolution fluorescence microscopy, we found that IDPs will passively diffuse through the NPC, independent of size, and will not strictly follow the rules of nucleocytoplasmic transport exhibited by folded proteins. Through a combination of native nuclear proteins and nuclear protein truncated we differentiated their diffusion efficiencies and routes by their content ratio of charged (Ch) and hydrophobic (Hy) amino acids. By using Ch/Hy-ratio to characterize IDPs, we can further our understanding of nucleocytoplasmic transport and how disease-related IDPs may exploit this new transport mechanism.

P539

Live cell reporters reveal bidirectional acceleration of nucleocytoplasmic transport by O-GlcNAc modification of the nuclear pore complex
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Macromolecular transport across the nuclear envelope is fundamental to eukaryotic cells and depends on facilitated diffusion through nuclear pore complexes (NPCs). The interior of NPCs contains a permeability barrier made of phenylalanine-glycine (FG) repeat domains that selectively facilitates the permeation of cargoes bound to nuclear transport receptors (NTRs). The NPC is enriched in O-linked N-acetylglucosamine (O-GlcNAc) modification, but its functional role in nucleocytoplasmic transport is unclear. We developed high-throughput assays based on optogenetic probes to quantify the kinetics of nuclear import and export in living human cells and showed that the O-GlcNAc modification of the NPC accelerates the nucleocytoplasmic transport in both directions. Super-resolution imaging of O-GlcNAc revealed strong enrichment at the FG barrier of the NPC channel. O-GlcNAc modification also promoted the passive permeation of a small, inert protein through NPCs. Our results suggest that O-GlcNAc modification accelerates nucleocytoplasmic transport by enhancing the non-specific permeability the FG-repeat barrier.

Establishing and Maintaining Organelle Structure: Lysosomes, Vacuoles and Exocysts

P540

The role of the exocyst member EXOC8 in the formation of cytoplasmic rods and rings
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Inosine monophosphate dehydrogenase (IMPDH) and cytidine triphosphate synthase (CTPS) are two rate-limiting enzymes involved in the catalysis of GTP and CTP. In humans, these enzymes can form into filamentous structures termed cytoplasmic rods and rings. Although cytoplasmic rods and rings have been described in multiple cell types, neither their formation nor their composition are well understood. Here we identify a novel localisation for the exocyst complex subunit EXOC8 to cytoplasmic rod/ring structures. Upon closer inspection, these structures resemble beads on a string, similar to the structure of IMPDH2-containing cytoplasmic rods and rings previously observed by cryo-EM. We found that EXOC8 interacts physically with IMPDH2 and CTPS1. Although it is not required to form IMPDH2-containing rods and rings in non-stressed cells, when these structures are induced by changes to metabolic conditions, rod length and organisation is altered in the absence of EXOC8. Mutations in the closely related enzyme IMPHD1 are a cause of inherited retinal degeneration, and mutation in EXOC8 is a candidate for pleiotropic syndromes where retinal dystrophy is part of the clinical presentation. We found that rod and ring localisation was lost in a disease-linked EXOC8 variant, and interaction with IMPDH2 was lost. This is the first time that an exocyst subunit - and indeed any membrane trafficking complex - has been linked to cytoplasmic rods and rings. We propose that the exocyst plays a role in trafficking proteins required for IMPDH2 rod and ring maturation and/or turnover, thereby regulating cyclic nucleotide metabolism, and that dysregulation may contribute to the molecular pathology of retinal degeneration and phototransduction.

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Investigating the effects of external stressors on lysosomal tubulation in macrophages

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Immune cells like macrophages can sample their environment for molecular clues for infection through endocytosis and/or engulf entire pathogens through phagocytosis. These cells use these foreign molecules as a code to initiate an attack to help resolve an infection. Additionally, lysosomes, which are highly acidic and degradative organelles, are essential to digest and clear the engulfed invaders. Interestingly, foreign molecules like bacteria-derived lipopolysaccharides (LPS) transforms lysosomes from globule-like structures to elongated tubules. This tubulation process is mediated by several well-known effectors like kinases Akt and mTORC1; however, the exact mechanism of lysosome tubulation and remains unclear. The low pH of lysosomes represents a H+ gradient that can also serve as a signal or energy source to drive other processes like molecular transport. To this end, I hypothesized that the lysosomal H+ gradient may be involved in reorganizing lysosome system in activated macrophages. I also postulated that the lysosome pH may change during this reorganization. To test these hypotheses, I manipulated the lysosome luminal pH through inhibition of the V-ATPase, which establishes the acidic pH of lysosomes. V-ATPase inhibition resulted in significant reduction in tubule formation and maintenance. However, the V-ATPase activity is also linked to mTORC1 activity, which is needed for tubulation. Indeed, Western blot analysis showed that drugs that block the ATPase activity of the V-ATPase decreased mTORC1 activity. To circumvent this issue, I am now using lysosomotropic bases like ammonium chloride and chloroquine, which neutralize the pH but allow V-ATPase to “pump”. Preliminary data suggests that this still inhibits tubulation, suggesting that the lysosomal pH is involved in re-organizing the lysosome system in macrophages. The project aims to demystify lysosomal dynamics in hopes of expanding the collective knowledge of this essential organelle.
Non-random distribution of vacuoles in *Schizosaccharomyces pombe*

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A central question in eukaryotic cell biology asks, during cell division, how is the growth and distribution of organelles regulated to ensure each daughter cell receives an appropriate amount. For vacuoles in budding yeast, there are well described organelle-to-cell size scaling trends as well as inheritance mechanisms involving highly coordinated movements. It is unclear whether such mechanisms are necessary in the symmetrically dividing fission yeast, *Schizosaccharomyces pombe*, in which random partitioning may be utilized to distribute vacuoles to daughter cells. To address the increasing need for high-throughput analysis, we are augmenting existing semi-automated image processing by developing fully automated machine learning methods for locating vacuoles and segmenting fission yeast cells from brightfield and fluorescence micrographs. All strains studied show qualitative correlations in vacuole-to-cell size scaling trends, i.e vacuole volume, surface area, and number all increase with cell size. Furthermore, increasing vacuole number was found to be a consistent mechanism for the increase in total vacuole size in the cell. Vacuoles are not distributed evenly throughout the cell with respect to available cytoplasm. Rather, vacuoles show distinct peaks in distribution close to the nucleus, and this preferential localization was confirmed in mutants in which nucleus position is perturbed. Disruption of microtubules leads to quantitative changes in both vacuole size scaling trends and distribution patterns, indicating the microtubule cytoskeleton is a key mechanism for maintaining vacuole structure.

Designing cell factories: The impact of vacuolar size on biochemical yield

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The organelles of eukaryotic cells house molecules involved in specialized biochemical reactions. We focus on the vacuoles of *Saccharomyces cerevisiae* yeast since they are dynamic organelles that have been suggested to hold the potential to manufacture biochemical products that can be converted to liquid fuels such as gasoline. The aim of this study is to determine if the sizes of these organelles impact the quantity of chemical products stored within them, which can provide insights on its capacity to yield compounds. Our compound of interest is 5-Aminoimidazole ribonucleotide (AIR), which accumulates in the vacuoles of *S. cerevisiae* mutants defective in the ADE2 gene (also involved in the DNPB pathway) when cells are grown in adenine-deprived medium. AIR emits red fluorescence naturally when exposed to oxygen, allowing us to measure the extent of its accumulation in vacuoles using fluorescent microscopy. We used spinning disk confocal microscopy to image AIR accumulation in ade2 mutants whose vacuoles were also labeled at the limiting membrane with a VPH1-GFP fusion. These images were used to quantify the three-dimensional size of the vacuoles using computational shape reconstruction as well as the AIR content from integrated fluorescence intensity. Our preliminary results suggest a positive correlation between vacuolar size and the amount of AIR accumulated. In addition, our results show that AIR accumulation is time-dependent and that it varies by cell. Understanding how vacuolar size affects the amount of a compound accumulated within the vacuoles could shed light on finding effective
ways to engineer yeast vacuoles as bioreactors to manufacture other compounds of interest in larger scales.

**Extracellular Vesicles**

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**Chemical and enzymatic treatments that promote cellular uptake of giant cell-derived extracellular vesicles**

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Plasma membrane blebs, the spherical protrusions formed on the cellular cortex, are one example of membrane dynamics on the cellular cortex. They can be observed not only during physiological situations, including cytokinesis, migration, apoptosis, and virus infection, but also during artificial stress conditions, such as chemical fixation using aldehyde fixatives and polar organic solvent and osmotic treatment. While giant membrane vesicles shed from membrane blebs on the cellular cortex have been used as membrane models for studying cell surface lipid and protein dynamics, their interactions and communication with cells remain largely unknown. The results from our study indicate that a large amount of giant cell-derived membrane vesicles (GCMVs), which appeared similar to giant plasma membrane vesicles originally prepared by Scott in 1976, were obtained during incubation of paraformaldehyde (PFA)-treated human cervical cancer HeLa cells in phosphate buffered saline. Exposure of PFA-treated cells to 5%-10% dimethyl sulfoxide also facilitated generation of GCMVs. Biochemical and microscopic analyses revealed the involvement of proteins and RNAs in the GCMVs and enabling visualization of the GCMVs using protein- and RNA-detecting fluorescent agents. On the basis of co-incubation experiments of HeLa cells and fluorescently labeled GCMVs, we showed that pre-treatment of cells with the glycosylation inhibitor tunicamycin or glycosylation digesting enzymes facilitated transfer of fluorescent signals from the GCMVs to cells. Our findings may be helpful for researchers who are interested in preparation and manipulation of cell-derived membrane vesicles by chemicals and enzymes, and suggest that the glycocalyx of recipient cells plays a role in the uptake of constituents present in GCMVs. 1) **Okada S**, Fukai Y, Yoshimoto F, Saitoh H. Chemical manipulations to facilitate membrane blebbing and vesicle shedding on the cellular cortex. Biotechnol Lett. 42:1137-1145 (2020). 2) **Okada S**, Yankawa S, Saitoh H. Wash-free instant detection of giant plasma membrane vesicles. Anal Biochem. 557:59-61 (2018). 3) **Okada S** and Saitoh H. in preparation (2020).

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**Effect of isoprenoid depletion on extracellular vesicles and miRNAs levels in cellular models of mevalonate kinase deficiency**

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Mevalonate Kinase Deficiency (MKD) is a rare autoinflammatory disorder caused by a recessively inherited mutation in the mevalonate kinase enzyme, which acts early in the isoprenoid biosynthetic pathway. Among the downstream molecules affected by isoprenoid depletion are Rab GTPases, which regulate membrane trafficking and extracellular vesicle (EV) secretion. EVs are known to deliver cellular
Cargo such as protein and RNA molecules to target cells, which can impact the inflammatory response. In addition, isoprenoid depletion is known to alter inflammatory signal transduction pathways, which may alter the molecules contained within the EVs such as miRNAs. In this study, we investigated how isoprenoid depletion affects EVs and miRNA levels from macrophages and monocytes. Cells were treated with lovastatin to deplete isoprenoids and an inflammatory response was induced by stimulation with lipopolysaccharide (LPS). EVs were isolated by precipitation and Nanoparticle Tracking Analysis (NTA) was used to determine EV quantity. RNA was isolated from cells and expression levels of LPS responsive miRNAs were measured by quantitative real-time PCR. Smaller vesicles in the size range of exosomes (30-100 nm) were decreased while larger vesicles in the size range of microparticles and apoptotic bodies (>100nm) were increased in the media of lovastatin as compared to carrier treated cells (24 h). In addition, the expression levels of miR-155, miR-9, miR-147b, and miR-204 were significantly lower in lovastatin as compared to carrier treated cells 24 hours following LPS stimulation. Co-incubation of lovastatin-treated cells with mevalonate prevented these changes indicating that they are the result of isoprenoid depletion. These results indicate that both the quantity of EVs and miRNA levels are altered by isoprenoid depletion, which may affect inflammatory pathways in macrophages and monocytes as well as other immune cells.

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A novel role of presynaptic periactive zone proteins in extracellular vesicle trafficking
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Extracellular vesicles (EVs) are small, endosomally-derived, membrane bound vesicles that can transport cargoes between cells, and are important for cell-cell communication in the nervous system. However, it remains unclear how specific endosomal cargoes are sorted for release through the EV pathway. Much of our knowledge of EVs comes from in vitro or cell culture-based studies, which are unable to fully recapitulate the process of endogenous EV cargo trafficking across complex tissues, such as the nervous system. To overcome these limitations, we have developed tools to use the Drosophila neuromuscular junction (NMJ) as a model system to study the trafficking of EV cargoes in vivo from the presynaptic neuron to the postsynaptic muscle. Through a directed genetic screen, we identified an unexpected role for presynaptic periactive zone (PAZ) membrane remodeling proteins, which have canonical roles in clathrin-mediated endocytosis, in regulating the traffic of EV cargoes at the Drosophila NMJ. PAZ mutants, including those lacking synaptojanin, dynamin, endophilin, and the F-BAR/SH3 protein Nervous Wreck (Nwk) exhibit a local and dramatic decrease in the levels of the EV cargoes Synaptotagmin-4 (Syt4) and Amyloid Precursor Protein (APP) at presynaptic terminals. Further, this decrease is sufficient to abolish Syt4 function and reduce APP toxicity, suggesting that loss of specific EV cargoes may play unrecognized roles in canonical phenotypes of PAZ mutants. Interestingly, this novel EV cargo traffic defect is genetically separable from the well-established functions of these proteins in synaptic vesicle recycling and synaptic growth. Our data suggests a novel clathrin-dependent molecular mechanism that protects EV cargoes from local degradation at synapses, and promotes their release and function in EVs.
G1, G1-S, and S Phase Regulation: Cell Cycle

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Cryo-Electron Microscopy Structure of the Human CDK-Activating Kinase

The human CDK-activating kinase (CAK) is a critical regulator of both transcription initiation and the cell cycle. A fine modulation of the cell cycle and tight control of gene expression are required for appropriate cell growth and faithful cell division. Failure of the mechanisms and checkpoints regulating these processes can lead to proliferative diseases. Because CAK impinges in both of these regulatory networks, it has been proposed as a potential drug target for cancer treatment. CAK is composed of the cyclin dependent kinase (CDK) 7, cyclin H and MAT1. The complex engages in cell cycle progression by phosphorylating the regulatory T-loop of some CDKs. We have obtained the three-dimensional structure of the catalytic module of human CAK at 2.8 Å resolution using cryo-electron microscopy (cryo-EM), revealing its architecture and providing insight into CDK7 activation. The structure shows that MAT1 acts as an assembly factor by substantially extending the interaction interface between the CDK7-cyclin H pair. Additionally, this unique third component of the complex makes contacts with the CDK7 T-loop, favoring an extended conformation of this loop, which may enhance CAK activity. With the aim of aiding the drug discovery process we have also solved the structure of THZ1, an anti-cancer small molecule inhibitor of CDK7, bound to a CAK truncated complex. Our structural characterization provides mechanistic insight into the role that CAK's trimeric assembly plays in transcription initiation and regulation of the cell cycle. From the technical perspective, our work also exemplifies how cryo-EM is a powerful technique for structure determination of a small (84 KDa), asymmetric complex at better than 3 Å resolution, paving the way for structure-guided design and improvement of molecular therapeutics.

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Three subunits of human ORC are dispensable for DNA replication in cancer cell lines, but the ORC holocomplex is important for both repressing and activating chromatin
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Structural studies suggest that Orc1 to Orc6 proteins form an ORC ring that with CDC6 protein interacts end-on-end with the MCM2-7+CTD1 ring to open the latter ring and enable it to encircle DNA. We tested whether cancer cells can replicate their DNA without specific subunits of ORC. We have reported that CRISPR/Cas9 mediated mutations in human cancer cell lines in both alleles of ORC2 or ORC1 make the corresponding proteins undetectable in the cells, and yet the cells still proliferate and replicate despite a significant decrease in chromatin loading of MCM2-7 (Shibata E, eLife, 2016). We now report that ORC5 loss, or the simultaneous loss of two subunits, ORC2 and ORC5, also permits cell proliferation, but without even a decrease in the chromatin loading of MCM2-7. The cells replicate with intact bidirectional origins of DNA replication. Although models of ORC action suggest that the six subunit ORC loads on the chromatin as a holo-complex to load CDC6 and CDT1+MCM2-7, chromatin loading of ORC6, CDC6 and CDT1 appear unaffected by the loss of ORC1, 2 or 5. Several of the remaining ORC subunits are
destabilized in the cells with mutation of ORC2 or ORC5, so that it is difficult to tell if the vanishingly small amounts of the remaining ORC subunits still associate with each other to form a functional subcomplex. Thus, although the six-subunit ORC complex is probably essential for chromosomal replication, it is possible for mutant cancer cells to bypass the strict requirement of ORC for chromosomal replication by a pathway that still requires CDC6. In contrast to the minimal effects on DNA replication initiation, there are extensive changes in the distribution of open chromatin in the ORC2−/− cells. Many sites that were formally in closed configuration become open in ATAC-seq experiments, consistent with the suggestion that ORC has an important role in establishing repressed chromatin domains. Surprisingly, there are also thousands of sites, mostly near promoters and at super-enhancers, where the loss of ORC leads to closed chromatin and decrease in expression from nearby promoters. The latter sites overlap significantly with Orc2 Chip-seq sites, suggesting that ORC also has a role in directly activating chromatin.

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**Pp2a(cdc55)-dependent degradation of cell cycle regulators, pds1 and swe1, is required for anaphase onset after replication stress**

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Accurate DNA replication is essential for genome stability. Stressful DNA replication causes DNA damage; the S-phase checkpoint pauses the cell cycle and preserves the functionality of replication forks in response to replication stress. However, it is poorly understood how cells recover from cell cycle arrest induced by DNA replication stress. Cyclin-dependent kinase (CDK) and Protein Phosphatase 2A (PP2A) are key regulators of cell cycle progression. Cdc55, one of the regulatory subunits of PP2A in budding yeast Saccharomyces cerevisiae, has previously been identified as a negative regulator of mitotic exit. We found that deletion of the PP2A catalytic subunits (Pph21 Pph22) or Cdc55 led to hydroxyurea (HU) sensitivity, a DNA synthesis inhibitor. Moreover, PP2A mutants exhibited sustained levels of anaphase inhibitor Pds1 along with delayed anaphase entry after treatment with HU, indicating that PP2A(Cdc55) is required for checkpoint recovery after DNA replication stress. In response to DNA damage, Pds1 becomes stabilized via phosphorylation by Chk1 (checkpoint kinase 1). Interestingly, chk1Δ and mutation of the Chk1 phosphorylation sites in Pds1 largely restore efficient anaphase entry in cdc55 mutants after HU treatment. In contrast, it appears that CDK-dependent Pds1 phosphorylation is not involved in this regulation. Previous studies show that PP2A(Cdc55) promotes the degradation of Swe1, a protein kinase that phosphorylates and inhibits mitotic Cdk1. We further showed that SWE1 deletion also suppresses the anaphase entry delay in PP2A mutants after HU treatment. Together, our results indicate that PP2A-dependent degradation of cell cycle regulators Pds1 and Swe1 allows efficient recovery from cell cycle arrest induced by DNA synthesis inhibition.

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**TICRR/TRESLIN binds to the nuclear matrix and is degraded in S-phase**

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A DNA replication program, which ensures that the genome is accurately and completely replicated, is established during the G1 phase of the cell cycle, prior to the onset of S-phase. In G1, replication origins are licensed by the loading of two minichromosome maintenance (MCM) heterohexamers onto chromatin, and upon S-phase entry, a subset of these will form active replisomes. In yeast, the replication initiation factor Sld3 marks the earliest firing replication origins during G1. However, in higher eukaryotes, the mechanisms by which the replication program is established in G1 are still unclear. Numerous studies suggest DNA replication occurs at the nuclear matrix (NM) where a proportion of pre-replicative complex (pre-RC) proteins including ORC, CDC6, CDT1, and MCMs can be detected during late G1. In addition, it has been demonstrated that there is an increase in mammalian replication origin sequences associated with the NM during late G1 and these decrease during S-phase. Sld3 is poorly conserved in eukaryotes, and little is known about its role in establishing the replication program in metazoans. Therefore, we aimed to define the chromatin/nuclear matrix association of the human Sld3 homolog, TICRR/TRESLIN, around the G1/S-phase transition. We tagged the endogenous C-terminus of TICRR with mClover in HCT-116 cells using CRISPR/Cas9. We applied an established flow cytometry based assay to detect levels of both insoluble and total TICRR and cell fractionation to determine if TICRR binds to chromatin or the NM throughout the cell cycle. We found that TICRR is enriched on the NM during early G1 independent of replication licensing and MCM-chromatin loading. Importantly, although the total expression level of TICRR decreases between G2/M and G1 phases, the level of NM-bound TICRR increases. This demonstrates that the increased TICRR binding to the NM in G1 is not due to changes in its expression. In contrast, both TICRR expression and the amount of NM-bound TICRR sharply decrease with S-phase entry and the onset of DNA replication through a proteasome-dependent mechanism. The recruitment of TICRR to the nuclear matrix during G1 and its degradation during S-phase are unexpected and may reveal important differences between replication initiation control in yeast and higher eukaryotes. Collectively, these results suggest an additional role for TICRR during G1 possibly in the recruitment and selection of origins to be activated in the ensuing S-phase.

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A double-assurance mechanism controls cell cycle arrest during invasive differentiation

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Cell invasion is a highly regulated cell behavior that occurs during normal embryonic development and becomes dysregulated during cancer progression and metastasis. To elucidate mechanisms of cell invasion, we use the in vivo model of Caenorhabditis elegans anchor cell (AC) invasion into the vulval epithelium. The AC is a terminally differentiated (TD) uterine cell that breaches the underlying basement membrane to orchestrate the uterine-vulval connection that occurs during larval development. Like many TD cells, the AC is arrested in the G0 phase of the cell cycle. We previously demonstrated that the AC is required to be in a G0 state in order to invade. We also discovered that nhr-67/Tlx mediates this AC cell cycle state in part through cki-1, a cyclin-dependent kinase inhibitor of the Cip/Kip family. However, the mechanism that maintains the AC in G0 arrest remains unclear, as loss of cki-1 alone is not sufficient to induce an AC proliferation phenotype. Here, we used RNA interference (RNAi) to individually deplete additional inhibitors of the G1/S transition, including lin-35, the sole homolog of Rb, cdc-14, a positive regulator of cki-1, and cki-2, a poorly characterized paralog of cki-1. Each RNAi experiment yielded lowly penetrant AC invasion and proliferation defects. Using our cyclin-dependent
kinase (CDK) sensor for \textit{in vivo} cell cycle state analysis, we found that loss of \textit{cki-1} in a \textit{cki-2} null mutant triggers a robust cycling AC phenotype. Together, our data demonstrate that loss of a single negative regulator of G1/S does not lead to AC proliferation, though loss of two G1/S inhibitors is sufficient to drive cell cycle entry. Thus, we hypothesize that a double-assurance mechanism controls AC cell cycle arrest. These results help to establish a model of cell cycle regulation during cell invasion, which is vital to developing cancer therapies that target cell cycle machinery.

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\textbf{Genetic interactions between \textit{pam-1} and \textit{wee-1.3} during oocyte maturation and meiosis in \textit{C. elegans}}

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In \textit{C. elegans}, the puromycin-sensitive aminopeptidase, PAM-1, is necessary for timely meiotic exit and proper establishment of the anterior-posterior axis. While this aminopeptidase is highly conserved, little is known of its targets in any species. In order to learn more about this, we conducted a suppressor screen and identified a missense mutation in \textit{wee-1.3} as a suppressor of \textit{pam-1}. While only 2\% of embryos laid by \textit{pam-1} mutant worms survive, the presence of the \textit{wee-1.3(lz5)} suppressor restores the hatch rate to approximately 50\%. WEE-1.3 is an inhibitory kinase that is involved in oocyte maturation through negative regulation of the MPF. To examine how WEE-1.3 and PAM-1 interact, we looked at oocyte maturation. Worms treated with \textit{wee-1.3(RNAi)} become sterile within 24 hours due to precocious oocyte maturation. However, \textit{pam-1} worms treated with the same RNAi are protected against this sterility and lay a comparable number of embryos to \textit{pam-1} mutants alone. In addition, precocious oocyte maturation is not observed after \textit{wee-1.3(RNAi)} treatment in \textit{pam-1} worms when oocyte maturation markers were examined. This interaction is not at the level of WEE-1.3 protein levels or localization, as they are comparable in wild-type and \textit{pam-1} worms. We are currently looking for interactions with the MPF and the CDC-25 phosphatase to learn more about this interaction in oocytes. In addition, we are examining meiosis in suppressed and unsuppressed embryos to see how WEE-1.3 and PAM-1 interact in this process. This work will lead to a better understanding of the role of this aminopeptidase in cell cycle regulation during oogenesis and meiosis. This work is funded by NIH 2R15GM110614-02.

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\textbf{Assessing Heterogeneity as Cells Enter and Exit the Endoreplication Cell Cycle}

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Endoreplication is an alternative cell cycle wherein cells go through alternating G and S phases without completing mitosis. While endoreplication is a normal process that can be used to generate natural polyploid cells, it can also be co-opted by cancer cells and lead to genome instability and tumor evolution. We have developed a method to generate induced endoreplicating cells (IECs) by treatment with cell cycle kinase inhibitors to define the mechanisms that allow entry into and exit from endoreplication. Inhibition of Aurora B kinase resulted in a type of endoreplication called endomitosis in which cells enter mitosis but do not complete division. Cells treated with higher concentration of inhibitor fail mitosis at the metaphase/anaphase transition, leading to the appearance of multi-lobed nuclei. In contrast, cells treated with lower concentrations of inhibitor fail at cytokinesis, resulting in
binucleated cells. These results suggest Aurora B inhibition prevents cells from completing mitosis in a dose-dependent manner. Furthermore, we have shown that cells with multi-lobed nuclei preferentially undergo DNA synthesis, suggesting the nuclear phenotype may play a critical role in determining whether a cell can bypass the tetraploid checkpoint. To ask how cells exit endoreplication, we generated iECs and used flow cytometry to isolate populations of cells with 2C/4C or 8C DNA and then allowed them to resume mitosis after drug washout. During outgrowth, we saw a lag in proliferation before cells began to rapidly divide and form colonies of mitotically dividing cells, suggesting that iECs are senescent prior to resuming division. To understand how iECs resume division and which iECs are capable of division, we focused our studies on the critical time period during which proliferation begins. We examined nuclear morphology at two-day time intervals during outgrowth. Initially, all of the cells had large multi-lobed nuclei, but over time we observed a small, but consistent increase in the proportion of binucleated cells. This suggested that binucleation may be an intermediate step between multi-lobed nuclei and the normal nuclear phenotype seen after outgrowth, and that cells may exit endoreplication using the reverse mechanism of how they entered. We also found a high instance of micronucleation amongst iECs and observed that the proportion of micronucleated cells decreased over time. This suggested that perhaps micronuclei are abolished from the cell in a nuclear budding-like manner. We are currently using immunofluorescent and time-lapse imaging to understand the heterogeneity of both entry into and exit out of endoreplication, with an ultimate goal of defining the molecular mechanisms necessary for these changes in cell cycle dynamics.

Integrin Signaling and Dynamics

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Loss of desmosomal cadherins enhances expression of extracellular matrix proteins.

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Desmosomes are protein complexes crucial for maintaining cell-cell adhesion and integrity of tissues. These complexes are made up of proteins from three families: transmembrane cadherins (Desmoglein and Desmocollin) link adjacent cells in the extracellular space, armadillo proteins Plakophilin (PKP2) and Plakoglobin (PG) stabilize the intracellular plaque, and the cytolinker Desmoplakin (DP) connects the plaque to the intermediate filament network. Desmosomal proteins have also been shown to coordinate gene expression pathways required for processes such as proliferation, differentiation and cell migration. In particular, several lines of evidence have linked desmosomal proteins to gene expression of extracellular matrix (ECM) proteins. Loss of PKP2 or DP causes increases in expression of fibronectin and collagen, while in contrast, loss of PG results in a significant decrease in expression of fibronectin. These data indicate that individual components of the desmosomal complex control ECM gene expression via distinct cellular signaling networks. In our study, we sought to investigate the role of desmosomal cadherins in ECM gene expression, via use of A431 cells lacking Desmoglein-2 (Dsg2 KO) or Desmocollin-2 (Dsc2 KO), generated via CRISPR-mediated knock-out. Compared to control cells, Dsg2 KO cells demonstrated a dramatic (~10-fold) increase in expression of Fibronectin (FN1), and relatively minor changes in expression of Collagen 1 (COL1A1) and Collagen 2 (COL2A1). Similar changes in expression of these ECM genes were also observed in Dsc2 KO cells. Increased expression of FN1, COL1A1 and COL2A1 was also observed upon siRNA-mediated knockdown of Dsg2 in A431 cells,
verifying that these changes are not clone-specific or due to off-target CRISPR effects. While expression of other desmosomal proteins is unchanged in Dsg2 KO cells, the integrity of the junctional complex was expectedly perturbed, as observed via a significant increase in Triton-solubility of PKP2, PKP3, PG and DP. Nevertheless, siRNA-mediated knockdown of these proteins could not rescue FN1 increases in Dsg2 KO cells, indicating that changes are not due to mis-localization of other desmosomal components.

Further investigation of signaling pathways known to regulate ECM gene expression have uncovered a role for Rho/SRF signaling in Dsg2-mediated control of FN1 gene expression. In contrast, inhibition of either Erk, p38 MAPK or TGF-beta signaling was insufficient to rescue the elevated expression of FN1 in Dsg2 KO cells. Taken together, our study highlights a novel role for Dsg2 in mediating ECM gene expression, adding significant insight into the mechanisms by which desmosomal cadherins control the adhesive behavior of cancer cells.

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**A Role for Myristoylated Alanine Rich C-Kinase Substrate (MARCKS) in Neutrophil Outside-in Beta2-integrin Activation and Signaling**

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**Objective:** MARCKS (Myristoylated Alanine Rich C-Kinase Substrate) is an actin and PIP2 binding protein that plays an essential role in primary neutrophil migration and adhesion; however, the molecular details regarding MARCKS function in these processes remains unclear. Neutrophil adhesion and migration also require beta2-integrins. Activation of beta2-integrins consists of a well-described sequence of events including increased cell surface expression (upregulation), change in conformation (affinity), clustering (avidity), and signaling. In this study, we hypothesize that MARCKS protein function plays a key role in one or more events required for neutrophil beta2-integrin activation. **Methods:** Isolated peripheral blood neutrophils were pretreated with MARCKS-specific inhibitor peptide MANS, scrambled control peptide RNS, or PBS (vehicle control). Flow cytometry was used to quantify surface expression of total CD18 (IB4) and CD11b (ICRF44), and high-affinity CD11b (CBRM1/5), in fMLF-stimulated and unstimulated neutrophils. A static, plate-based assay was used to quantify ICAM-1 induced neutrophil adhesion. Confocal microscopy and immunofluorescence were used to examine ICAM-1 induced neutrophil spreading and beta2-integrin clustering. DHR fluorescence was used to quantify PMA vs. insoluble immune complex (IIC)-stimulated respiratory burst. **Results:** MARCKS inhibition with the MANS peptide significantly attenuated outside-in beta2-integrin adhesion, spreading, and clustering on ICAM-1; as well as beta2-integrin independent IIC-stimulated respiratory burst. MANS pretreatment did not affect fMLF-induced upregulation or conformation change of beta2-integrins or beta2-integrin independent PMA-stimulated respiratory burst. **Conclusion:** We conclude that MARCKS function is essential for beta2-integrin outside-in, but not inside-out, activation in human neutrophils in vitro. This is the first report of a role for MARCKS in outside-in activation of neutrophil beta2-integrins.

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**Desmoglein-2 modulates cell spreading and focal adhesion dynamics via Rap1 and TGF-beta signaling.**

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The desmosome is a cell-cell adhesion complex which facilitates the mechanical stability of tissues and cell-cell communication. Desmosome function depends upon a tripartite organizational structure wherein transmembrane cadherins (Desmoglein and Desmocollin) link adjacent cells in the extracellular space, armadillo proteins (Plakophilin and Plakoglobin) stabilize the intracellular plaque, and the cytolinker Desmoplakin (DP) connects the plaque to the intermediate filament network. In addition to their central role in maintaining cell-cell junction integrity, desmosomal cadherins also coordinate biological processes such as proliferation, apoptosis, differentiation and cell migration. In our study, we sought to investigate the signaling mechanisms involved in control of actin architecture via desmosomal cadherins, using A431 cells lacking Desmoglein-2 (Dsg2 KO), generated via CRISPR-mediated knock-out. Wildtype and Dsg2 KO A431 cells were subjected to single cell spreading assays on different extracellular matrix proteins. Compared to control cells, Dsg2 KO cells displayed a significant increase in spreading area on both fibronectin and collagen. As these experiments were performed in singly spreading cells, these experiments have identified a novel cell-autonomous, cell-cell adhesion-independent role for Dsg2 in regulation of cell spreading. Spreading changes in Dsg2 KO cells were dependent on Rap1 GTPase, as siRNA-mediated knockdown of Rap1 in Dsg2 KO cells rescued the increase in spreading area. Dsg2 KO cells also demonstrated a Rap1-dependent increase in phosphorylation of FAK and Paxillin, suggesting that the enhanced spreading phenotype may be due to alterations in integrin-dependent cell-matrix attachment. Changes in Dsg2-mediated cell spreading was also shown to require the Rap GEF PDZ-GEF2 (but not PDZ-GEF1 or other Rap GEFs). Further investigation into signaling pathways known to affect cell spreading identified a role for TGF-beta signaling, but neither Erk or p38 MAPK were involved. Interestingly, Dsg2 KO cells demonstrate significantly elevated production of TGF-beta2 (but not TGF-beta1 or 3), and inhibition of TGF-beta receptor signaling via SB431542 rescued the increase in spreading area and enhanced phosphorylation of focal adhesion proteins seen in Dg2KO cells. These data have therefore identified a novel cell-cell adhesion independent role for Desmoglein-2 in mediating cell spreading via Rap1 and TGF-beta signaling, which provides significant insight into the signaling mechanisms via which desmosomal cadherins control cell-matrix attachment and cytoskeletal architecture.

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Super-resolution microscopy reveals nano-hubs of spatially segregated proteins within focal adhesions.
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Focal Adhesions (FA) are mechanosensitive complexes that connect the extracellular matrix (ECM) with the actin cytoskeleton. This is achieved through clustered integrins, their ligands in the ECM, and an internal dynamic protein complex linking the cytosolic domain of integrins with the actin cytoskeleton. In contrast to the established view that FAs are homogeneous micron-scale protein assemblies, recent super-resolution imaging and single molecule dynamic approaches are challenging this view. These studies suggest that FA molecular components are highly organized in the axial direction establishing segregated layers of functional activity. Recent data also indicates that similar type of nanoscale modularity might exist in the horizontal plane of FAs. Here, we present a set of experiments aimed at dissecting the lateral nanoscale organization of different FA proteins. Employing dual-color super-resolution microscopy, STORM, we reveal that common proteins associated to FAs, such as paxillin, talin
and vinculin, are organized in segregated nanoclusters within FAs. Whereas paxillin nanoclusters are mostly concentrated inside FAs, both vinculin and talin nanoclusters were found inside and outside mature FAs, albeit at different densities and molecular packing. Paxillin and vinculin nanoclusters inside FAs spatially segregate from each other at around 100 nm, while talin nanoclusters are more sparsely distributed and exhibit a larger variation in nanocluster sizes and molecular densities. Our results indicate that, in contrast to the canonical view that integrins within FAs actively engage with their main partners, paxillin, talin and vinculin, there exists a physical segregation between these molecules, forming nano-hubs at the nanoscale. Indeed, the larger heterogeneity in the nanocluster characteristics for talin is fully consistent with recent findings indicating that transient active vs. inactive integrin nanoclusters might reside within FAs. We further investigated the nanoscale organization and spatial distribution of $\alpha_\beta_1$ and $\alpha_\beta_3$, the two central integrins associated to FAs. Similar to their partners, integrins also organize in nanoclusters. $\alpha_\beta_1$ is more associated to fibrillar adhesions, whereas $\alpha_\beta_3$ nanoclusters are more abundant in FAs. Interestingly, while the distribution of $\alpha_\beta_1$ nanoclusters inside FAs appears quite random, $\alpha_\beta_3$ nanoclusters inside FAs locate particularly at the edges of FAs which are highly enriched by talin. The precise role of these two integrins within FAs has remained enigmatic. Our current hypothesis is that the difference in the distribution of these two mechanosensitive players is linked to their different roles in adhesion and in mechanotransduction.

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**Faster binding kinetics of low-affinity integrin conformations facilitates force-regulated adhesion**

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Integrin conformational ensemble contains two low-affinity states, bent-closed and extended-closed states, and a high-affinity, extended-open state competent for mediating mechanotransduction. Besides affinity, behaviors of adhesion molecules are also accounted for by binding kinetics. Here we characterized the intrinsic ligand-binding kinetics of each state in integrin $\alpha\beta_1$ and $\alpha\beta_3$. Notably, low-affinity states bind substantially faster than the high-affinity state. Slow ligand-association kinetics of the high-affinity state is compensated by 50,000-fold slower dissociation. Our results support an induced fit model of integrin-ligand interaction, wherein ligand binds more efficiently to the low-affinity conformations to form the closed complex, followed by a fast transition to the extended-open complex, which is stabilized by tensile force and has a long lifetime that enables prolonged lifetime even at high force. Rapid ligand-association kinetics of the low-affinity states allows integrin adhesiveness to be regulated after ligand binding. Tensile force transmitted through the integrin stabilizes the extended conformation, while ligand stabilizes the open conformation, resulting in the extended-open state.

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**Local calcium influxes reinforce lamellipodia protrusions by facilitating actin-adhesion coupling**

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Cell motility is an essential process in which cells migrate in response to various environmental cues. Many of the body's functions, such as wound healing and immune response, are reliant on the cells’ ability to control and coordinate the molecular machineries that drive cell migration. Networks that regulate actin cytoskeleton dynamics and focal adhesion complexes play a key role in cell migration, as...
they aid in the formation of lamellipodia, a specialized actin-based engine that pushes on the plasma membrane to create protrusions which drive the cell forward. However, the key molecular players within these networks have not been fully elucidated. Recently, it has been shown that the signaling ion, Ca²⁺, may be important for cell migration, but its precise spatial and temporal localization is yet to be properly defined. To visualize Ca²⁺ dynamics in migrating cells, we employed a genetically encoded Ca²⁺ sensor, GCaMP6f, and examined the distribution of Ca²⁺ in cellular protrusions. Kymographic analysis of protrusion dynamics revealed transient local influxes of Ca²⁺ in cell lamellipodia that coincided temporally with protrusions. We pharmacologically perturbed various sources of intracellular Ca²⁺ and determined that the local influxes of Ca²⁺ were maintained by stretch activated Ca²⁺ channels. To understand whether lamellipodia Ca²⁺ influx plays a regulatory role in protrusions, we employed a cell-spraying assay and found that inhibition of stretch activated Ca²⁺ channels slows lamellipodia protrusions. Furthermore, we screened a panel of 18 siRNAs targeting Ca²⁺-permeable channels and identified a single stretch activated channel, TRPV4, which is essential for protrusion efficiency. To understand the mechanism by which TRPV4-mediated Ca²⁺ influx regulates protrusions, we compared the dynamics of the actin cytoskeleton and integrin-based adhesions in lamellipodia of control and TRPV4-inhibited cells. We showed that inhibition of TRPV4 suppresses the assembly of integrin-based adhesions and decreases the mechanical coupling of the lamellipodia actin cytoskeleton to the extracellular matrix, impairing protrusion formation. Together these data reveal how TRPV4-mediated local Ca²⁺ influx regulates actin-adhesion coupling to reinforce lamellipodia protrusions.

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Cell adhesion and mechanotransduction at filopodia tips
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The extension of membrane protrusions is a prominent morphological feature during many cellular processes and serves as an important mechanism to probe the extracellular space and ascertain the appropriate cellular response. Filopodia are adhesive cellular protrusions specialized in recognition of the surrounding extracellular matrix (ECM). To this end, filopodia contain cell-surface receptors such as integrins that can interact with, and interpret, a wide variety of cues including ECM topography or stiffness. At focal adhesions, ECM sensing involves controlled integrin-activation as well as the recruitment of hundreds of proteins (“adhesome”) to tune cellular responses. In comparison, very little is known on how cell ECM-adhesion is regulated in filopodia. To understand how filopodia adhesions sense the ECM, we performed a structured-illumination-microscopy (SIM)-based screen to map the localization of 80 target proteins, linked to cell adhesion and migration, within filopodia. Our mapping reveals that filopodia adhesions consist of a unique set of proteins, the Filopodome, that are distinct from classical nascent adhesions or focal adhesions. Using live imaging, we observe that filopodia adhesions can give rise to nascent adhesions, which, in turn, form focal adhesions. Using fluctuation-based traction force microscopy, we find that filopodia typically align to the force field generated by focal adhesions indicating that these two structures are mechanically connected. Next, we thought to elucidate how integrins are regulated in these unique adhesion complexes. Using SIM and surface electron microscopy, we observed that integrin activation is spatially controlled in filopodia, with active integrin accumulating at filopodia tips while inactive integrin can be found throughout the filopodia shaft. RNAi depletion of integrin regulators identified FERM domain-containing talin and MYO10 as
critical regulators of filopodia function. Importantly, deletion of MYO10 FERM ablates the active pool of integrin from filopodia, indicating that MYO10-FERM domain is required for integrin activation but not for integrin transport to filopodia tips. Yet, remarkably, the MYO10-FERM domain binds both α and β integrin tails restricting integrin activation. Swapping MYO10-FERM with talin-FERM leads to an over-activation of integrin receptors in filopodia. Our observations demonstrate a complex regulation of integrin activity, at filopodia tips, via MYO10-FERM domain and challenge the concept of MYO10-dependent integrin transport in filopodia. We are now using these results to build a comprehensive model of how cell adhesion is regulated within filopodia to direct cell migration in complex environments.

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**Syndecan-4 regulates the expression of fibronectin and its receptor, alpha 5 beta 1 integrin (α5β1), in anoikis resistant endothelial cells**

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**Aims:** Our previous results demonstrated that the acquisition of resistance to anoikis positively regulates syndecan-4 (SDC4) and induce the remodeling of the extracellular matrix in endothelial cells, decreasing the expression of fibronectin and increasing the expression of its receptor α5β1 and its regulatory metalloproteinases (MMPs 2 and 9). In addition, we recently demonstrated that SDC4 silencing led to downregulation of proliferative, invasive and angiogenic abilities and an increase in adhesiveness of anoikis-resistant endothelial cells. Thus, this study investigated the interaction of syndecan-4 with fibronectin as well as with its receptor and regulators. **Methods:** Anoikis-resistant endothelial cells (Adh1-EC) transfected with micro RNA interference (miR RNAi ) targeted against syndecan-4 (miR-Syn-4-1-Adh1-EC e miR-Syn-4-2-Adh1-EC) were studied in comparison with parental anoikis-resistant rabbit aorta endothelial cells (Adh-EC), wild rabbit aorta endothelial cells (EC) and rabbit aorta endothelial cells transfected with the EJ-ras oncogene (EJ-ras EC) in relation to: fibronectin and α5β1 integrin expression, as well as MMPs 2 and 9 expression and activity. The gene and protein expression were analyzed by qPCR and Western blotting, respectively, and MMPs activity by zimography. **Results:** The results found showed that SDC4 gene silencing led to a decrease in α5β1 integrin expression, as well as in the expression and activity of MMPs 2 and 9. In contrast, it induced an increase in fibronectin protein. **Conclusion:** The downregulation of fibronectin expression and overexpression of α5β1, and MMPs are associated with a more malignant phenotype. Our results suggest that SDC4 overexpression plays an important role in remodeling the matrix of endothelial cells resistant to anoikis, increasing the degradation of fibronectin, affecting the binding of fibronectin to α5β1 integrin and consequently decreasing cell adhesion of anoikis resistant endothelial cells. The downregulation of syndecan-4 alter the malignant phenotype of these cells.

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**Cell matrix adhesions can be targeted by intracellular delivered antibodies**

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Cell-matrix adhesions contribute in defining the overall tissue architecture and are remodeled in numerous pathological conditions. Targeted modulation of cell-matrix adhesions in vitro can be achieved by changing the properties of cell or its environment. Cellular properties can be readily modified by RNAi or CRISPR-Cas9 gene editing, however, both methods interfere with the DNA- or RNA-based levels of regulation. In order to directly target proteins regulating cell-matrix adhesion and minimize side effects, we have developed protein transfection method. In contrast to the available approaches that require additional instrumentation or drastically interfere with cellular homeostasis, our method has no negative impact on cell viability and no specialized equipment is needed. We show, that antibodies that target intracellular regulators of integrin-mediated cell adhesion, Talin1 and Kindlin2, remain functionally active and exert their function post-transfection. We demonstrate that cell spreading and adhesion are effectively modified under these conditions. Antibody-based loss-of-function of Talin1 and Kindlin2 is as efficient as RNAi and gene editing, but supersedes these methods in terms of specificity. We are probing the method to specifically modify other integrin-mediated cellular events and beyond.

Mechanotransduction in Tissue Development and Morphogenesis

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**Mechanical Compartmentalization of the Intestinal Organoid Enables Crypt Folding and Collective Cell Migration**

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Intestinal organoids capture essential features of the intestinal epithelium such as folding of the crypt, spatial compartmentalization of different cell types, and cellular movements from crypt to villus-like domains. Each of these processes and their coordination in time and space requires patterned physical forces that are currently unknown. Here we map the three-dimensional cell-ECM and cell-cell forces in mouse intestinal organoids grown on soft hydrogels. We show that these organoids exhibit a non-monotonic stress distribution that defines mechanical and functional compartments. The stem cell compartment pushes the ECM and folds through apical constriction, whereas the transit amplifying zone pulls the ECM and elongates through basal constriction. Tension measurements establish that the transit amplifying zone isolates mechanically the stem cell compartment and the villus-like domain. A 3D vertex model shows that the shape and force distribution of the crypt can be largely explained by cell surface tensions following the measured apical and basal actomyosin density. Finally, we show that cells are pulled out of the crypt along a gradient of increasing tension, rather than pushed by a compressive stress downstream of mitotic pressure as previously assumed. Our study unveils how patterned forces enable folding and collective migration in the intestinal crypt.
Mechanosensitive mechanism of angiogenesis and lung regeneration through endothelial YAP1
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Pulmonary artery (PA) pressure and shear stress increase in the lungs after unilateral pneumonectomy (PNX). Mechanosensitive transcriptional co-activator, yes-associated protein (YAP1), in endothelial cells (ECs) is necessary for compensatory lung growth after PNX. However, the mechanism by which increases in PA pressure and shear stress following PNX control post-PNX lung growth through endothelial YAP1 remains unclear. Laminar shear stress increases the expression of Krüppel-like Factor (KLF2) in human pulmonary arterial ECs (HPAECs), which induces angiogenic factor Tie2 expression and stimulates EC DNA synthesis and migration. YAP1 WW domain mutant, which fails to bind to KLF2, accelerates Tie2 expression under shear stress. Hydrostatic pressure increases the expression of YAP1, induces Tie2 expression, and stimulates angiogenic activities in HPAECs, while knockdown of YAP1 or YAP1S94A mutant, which fails to bind to transcription factor, TEAD1, inhibits pressure-induced Tie2 expression and angiogenic activities. Ligation of a right cardiac lobe PA after left PNX stimulates PNX-induced increases in PA pressure and shear stress, upregulates expression of YAP1, KLF2, and Tie2 in lung ECs, and promotes post-PNX lung growth in the remaining non-occluded lobes. These effects are attenuated in endothelial-specific Yap1 knockout mice. Gene enrichment analysis further confirms that the levels of mechanosensitive genes are altered in ECs isolated from mouse lungs after PNX. Modulation of the mechanical environment or endothelial YAP1 signaling could be an efficient strategy for regenerative lung growth.

Cooperation between α-Catenin and its binding partners Vinculin, α-Actinin, Ajuba, and Afadin in Drosophila cell adhesion and morphogenesis
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Cell-cell contacts known as adherens junctions (AJs) must form both robust yet dynamic interactions in order for cells to remain tightly bound to each other throughout morphogenesis. Adhesion and epithelial integrity depend on the physical link between F-actin and the AJs provided by α-catenin, which is also thought to act as a mechanosensor - able to transduce mechanical input into downstream biochemical signals. Actomyosin contraction applies tension to the molecule, stretching the α-catenin M-region into an open conformation and revealing cryptic binding sites for actin binding proteins such as Vinculin. This interaction is thought to reinforce adhesion in the face of increased actomyosin forces. However, Vinculin null mutants, along with mutants for several other α-catenin binding partners such as α-actinin and Ajuba, have only subtle or no developmental defects. It is therefore unclear what role α-catenin mechanosensing plays in vivo for development or maintenance of an epithelium. Using live imaging and image analysis of Drosophila mesoderm invagination and germ band extension we aim to investigate the role of α-Catenin mechanosensing in supporting adhesion during morphogenesis. Here we report evidence of a substantive requirement of the α-catenin M-region to epithelial integrity in the early Drosophila embryo. Surprisingly, a smaller M-region deletion which exposes the Vinculin binding domain (M1) rescues α-Catenin knockout embryos more poorly than the full M-region deletion. The M1 domain appears to have an inhibitory effect on E-cadherin junctional stability, although not due to
loss of Vinculin recruitment. Furthermore, we found a striking genetic interaction between the M-region and its reported binding partner Afadin (Canoe) implying that Canoe and the M-region act in parallel. Our data begin to uncover the logic of the α-catenin-F-actin interface as seen through in vivo analysis, and support the view of cooperation and redundancy between α-Catenin and its interaction partners.

P566

Myosin motors regulate Drosophila stretch receptors

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Insects sense vibrations and body movements with chordotonal organs, specialized stretch receptors that monitor relative motion between body parts. We have performed a combination of electrophysiological and micromechanical experiments on living lch5 organs of Drosophila larvae. These chordotonal organs are pre-tensioned by accessory cap cells. We found that the extracellular matrix surrounding the cap cells maintains the basic resting tension. The extremely elastic cap cells contain microtubules, actin structures, and nonmuscle myosin-II motors. We found that myosin-II motor activity drives cap cell contraction and is involved in sensory adaptation. Optogenetic activation of myosin-II in the cap cells induced contractions and triggered spiking responses of the mechanoreceptors. Cap cell-specific knockdown of the regulatory light chain of myosin-II lowered tension in the chordotonal organs, decreasing the cap cell elastic modulus. Along with these mechanical effects, mechanoreceptor responses became more tonic, reflecting alterations in spiking synchronicity and mechanosensory adaptation. In addition to implicating myosin motors in insect mechanosensation, these findings document that, in chordotonal organs, two active machineries exist: chordotonal organs boost their mechanical input with ciliary dynein motors, and they employ myosin motors to adjust tension through cap cell contractility.

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Visualizing the Force Transmission Pathway in C. elegans Touch Combining Optogenetics and Tension Sensors

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Mechanical forces carry important information about our environment (e.g. during touch and hearing), and our own bodies (e.g. proprioception and cellular mechanosensing). In recent years, we obtained a deep understanding about the ion channels and receptors that convert the physical into a physiological signal. However, it is less clear how mechanical stresses actually reach the molecular mechanosensor. Whereas many mechanosensitive ion channels have been shown to be gated by increases in plasma membrane tension, evidence accumulates that emphasize the importance of the cytoskeleton within the sensory cell. Here, we use the model organism Caenorhabditis elegans to study the mechanotransduction pathway during gentle body touch, which is mediated by the six touch receptor neurons (TRNs) that express highly conserved mechanosensitive ion channel MEC-4 of the DEG/ENaC family (Katta et al., 2015). We first isolated TRNs from embryos and generated primary cultures with the aim to apply increasing membrane tension gradients using dynamic tether extrusion experiments in an
optical trap assay but failed to detect mechanosensitive activity up to forces that reach close to the lytic membrane tension (1mN/m). We then investigated the long-standing hypothesis that stomatin/MEC-2, the obligatory partner of MEC-4, associates with the cytoskeleton through a stereotypic SH3 binding motif present at its C-terminus (Huang et al., 1995). After screening ten different candidate binding partners that are naturally expressed in TRNs using a cellular competition assay, we found that UNC-89, a protein with high homology to human titin, colocalizes to the ion channel complex and, if overexpressed, interferes with the sense of touch. We next showed that this protein-protein interaction motif on MEC-2 is critical for mechanosensation, but dispensable for its localization to the pore-forming subunit of the ion channel complex, suggesting that MEC-2 has functions in direct force transfer. To test this, we engineered a molecular tension sensor module (TSMod) into MEC-2 and found that it is reversibly set under tension when a force is applied to the cuticle within a microfluidic device that mimics eternal touch. We will next study a possible interaction between MEC-2 and UNC-89 by biochemistry. Collectively, we suggest that a neuronally expressed titin might have yet undiscovered roles in force transmission during mechanosensation.

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Mechanochemical control of epidermal stem cell divisions by B-plexins

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The precise spatiotemporal control of cell proliferation is key to the morphogenesis of epithelial tissues. Epithelial cell divisions lead to tissue crowding and local changes in force distribution, which in turn suppress the rate of cell divisions. However, the molecular mechanisms underlying this mechanical feedback are largely unclear. Here, we identify a critical requirement of B-plexin transmembrane receptors in the response to crowding-induced mechanical forces during embryonic skin development. Epidermal stem cells lacking B-plexins fail to sense mechanical compression, resulting in disinhibition of the transcriptional coactivator YAP, hyperproliferation, and tissue overgrowth. Mechanistically, we show that B-plexins mediate mechanoresponses to crowding through stabilization of adhesive cell junctions and lowering of cortical stiffness. Finally, we provide evidence that the B-plexin-dependent mechanochemical feedback is also pathophysiologically relevant to limit tumor growth in basal cell carcinoma, the most common type of skin cancer. Our data uncover a central role of B-plexins in mechanosensation to couple cell density and cell division in development and disease.
Mechanotransduction and alternative splicing regulation in skeletal muscle cells
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Skeletal muscle is a mechanosensitive tissue that exhibits high levels of alternative splicing. RNA-binding proteins (RBPs) regulate alternative splicing by binding to motifs within pre-mRNA. Numerous genes encoding for force transmission and membrane trafficking proteins are alternatively spliced during muscle development, but the regulators are unknown. Defects in the mechanical properties of muscle and alterations in splicing lead to devastating muscular dystrophies. We aim to define how splicing is regulated by RBPs and how splicing of membrane trafficking genes is involved in determining the mechanical properties of muscle cells. During C2C12 skeletal muscle cell differentiation we found splicing of many trafficking genes was controlled by two RBPs, polyrymidine tract binding protein 1 (PTBP1) and quaking (QK) but not other RBPs (MBNL1, CELF1, ELAV1). PTBP1 is upregulated in myoblasts and promotes exon skipping by binding to TCTCT motifs. QK is slightly upregulated in myotubes and promotes exon skipping or inclusion depending on if it binds to ACUAA motifs upstream or downstream of the alternative exon. To further validate the involvement of PTBP1 and QK in splicing of membrane trafficking genes, we bioinformatically determined the presence of PTBP1 and QK motifs within and around membrane trafficking genes. We saw that motif location for PTBP1 or QK corresponded with the action of the RBP on the splicing of individual events. We also analyzed enrichment of PTBP1 or QK motifs between differentially spliced genes during development and found that specific locations around alternative exons were enriched for PTBP1 and QK motifs. Thus these two proteins work in concert to regulate membrane trafficking genes during muscle cell differentiation. PTBP1 and QK regulate the splicing of the capping actin protein of muscle z-line subunit beta (Capzb). CAPZB protein localizes to the z-discs in muscle where force is generated and caps growing actin filaments. To understand the function of this protein in C2C12 cells, we depleted CAPZB and determined the effect on cell proliferation and differentiation. We observed reduced proliferation and differentiation demonstrating that CAPZB is necessary for myogenesis. We next measured membrane stiffness of differentiated cells lacking CAPZB using atomic force microscopy. Cells lacking CAPZB exhibited different stiffness compared to controls leading us to hypothesize that CAPZB might contribute to membrane stiffness via capping actin filaments. In the future, we plan to determine the function of the alternative exon of CAPZB in skeletal muscle cells. Muscular dystrophies exhibit both splicing and mechanical alterations so linking an alternatively spliced gene with an impact on mechanical properties of muscle cells would be novel.

Keratin Intermediate Filaments Regulate GLUT2 Membrane Localization in Murine Pancreatic β-cells
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Introduction Among the hallmarks of diabetes is the impairment of glucose-stimulated insulin secretion (GSIS) which starts by the uptake of glucose via the glucose transporter 2 (GLUT2). Loss of GLUT2 is associated with hyperglycemia and impaired GSIS in several diabetes mouse models. Keratin 8 and 18
(K8/K18) intermediate filaments (IF), known to be involved in signaling and protein targeting, are expressed as the main IF proteins in pancreatic β-cells. Mice with global loss of K8 (K8−/−) show impaired β-cell function through defective mitochondria and alterations in insulin vesicle content and morphology. However, these mice have higher glucose tolerance and increased sensitivity to insulin, implying the involvement of other organs such as the liver in the glucose metabolism phenotype. Previously, we have shown the mislocalization of K8−/− β-cell GLUT2 to the cytoplasm compared to the plasma membrane localization seen in K8 wild-type (WT) mice, while GLUT2 levels were not altered. The K8−/− GLUT2 mislocalization correlates with a delay in streptozotocin (STZ)-induced diabetes (type I diabetes model), likely due to a decreased uptake of STZ which utilizes GLUT2 for β-cell entry. **Objective** To investigate the β-cell autonomous K8 function without interfering factors from other organs involved in glucose metabolism, we have developed a recombinant mouse model (Creins/FlFlK8) in which K8 is deleted only in β-cells. The objective of the study is to investigate if K8 in a β-cell autonomous fashion regulates GLUT2 plasma membrane localization and if intact keratin filaments are essential for this process. **Methods** A β-cell specific keratin knockout (Creins/FlFlK8) mouse is produced by crossing floxed K8-knockout mice with an insulin-CRE line. Pancreatic sections were analyzed with respect to GLUT2 localization. Murine insulinoma MIN6 β-cells transfected with WT K8/K18 or filament-disrupting K8/K18R90C plasmids, were analyzed with respect to GLUT2 localization basally and after treatment with STZ. **Results** Lack of K8 in β-cells of Creins/FlFlK8 mice leads to loss of membrane-proximal localization of GLUT2 and increased accumulation of GLUT2 in the cytoplasm compared to WT control (FlFlK8), which endorses our previous findings on GLUT2 localization in global K8−/− mice. Similarly, WT K8/K18 filaments over-expressed in MIN6 cells, promote membrane localization of GLUT2, while over-expression of filament-disrupting K8/K18R90C or cells with negligible K8/K18, have a disrupted membranous localization of GLUT2. MIN6 cells overexpressing WT K8/K18 depict a more cytoplasmic GLUT2 localization under STZ treatment compared to untreated control. **Conclusion** Our results indicate that intact K8/K18 filaments are needed to maintain membrane localization of GLUT2 in β-cells.

**Mitochondria and Cellular Metabolism**

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**Adipose mitochondrial metabolism couples nutrients to systemic insulin signaling and growth**

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The larval fat body (equivalent to the vertebrate adipose tissue and liver) is central regulator of whole-body physiology and growth in *Drosophila*. In nutrient-rich food, activation of TOR pathway in the fat body promotes endocrine signaling to the brain leading to the release of insulin-like peptides (ILPs) causing increased systemic insulin and growth. In contrast, in low nutrients, this insulin signaling is reduced and growth is decreased. An important question is how nutrients control fat body to mediate these effects on endocrine signaling and growth. Here we describe a role for mitochondria in this process. We found that in rich nutrients, fat body mitochondria were large with sparse cristae, and levels of both TFAM, a transcription factor which controls expression of the mitochondrial genome, and OxPhos activity were low. However, when larvae were switched to low nutrient food, which delayed growth and development, TFAM levels and OxPhos activity were increased and fat body mitochondria became smaller with dense cristae. To explore the significance of this finding we used RNAi to
knockdown TFAM, which lead to reduced OxPhos activity. We saw that fat body specific TFAM RNAi caused an acceleration of growth and development in rich nutrients, and was sufficient to reverse the delay in development seen in low nutrient food. These effects were accompanied by reduced fat body expression of Eiger/TNF-alpha, a negative regulator of brain ILP expression, and Imp-L2, an inhibitor of insulin signaling, and reduced whole-body expression of FOXO target genes. These effects are consistent with an increase in systemic insulin signaling. Also, we found that fat body TFAM RNAi increased expression of key glycolytic genes. Based on these findings we propose that a mitochondria-mediated switch in adipose OxPhos vs glycolytic metabolism can couple nutrient availability to fat-body mediated changes in systemic insulin signaling and growth. Research funding sources: Canadian Institutes of Health Research (CIHR) and Cancer Research Society (CRS).

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Characterizing the effects of metabolic toxins on cellular oxygen consumption
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Treatment for chemical exposure often centers on supportive care. To identify more targeted treatments for metabolic toxins, we developed cellular models to screen therapeutic countermeasures. While significant research has explored the cellular effects of exposure, further study is required to better understand and treat the underlying mitochondrial dysfunctions. Two toxins, fluoroacetate and thallium, both lack therapeutics that effectively address cellular dysfunctions resulting from exposure. Employing Agilent’s Seahorse XF platform, we developed in vitro models which characterize chemical intoxication on a cellular level. We also characterized innate cellular responses to chemical exposure, including activation of alternative energy pathways. These data suggest that screening specific classes of therapeutics such as antioxidants may be a useful strategy to counter the effects of intoxication on the cellular level. Alternatively, in vitro data exploring the innate cellular response to these chemicals suggest screening therapeutics that support alternative energy pathways may allow cells to circumvent compromised mitochondrial function. Specifically, these assays identified significantly decreased maximal respiration in response to fluoroacetate poisoning. We observed decreased dependence on glucose metabolism and increased dependence on fatty acid oxidation, suggesting a targeted treatment strategy that supports alternative energy pathways. Thallium more subtly induces decreases in ATP production and maximal respiration along with increased non-mitochondrial oxygen consumption.

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LKB1 maintains metabolic compartmentalization and metabolic efficiency in the hepatic lobule
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The liver is a metabolic organ that senses and coordinates feeding and fasting responses in order to maintain energy homeostasis. The fasting response is partly mediated through the metabolic sensor liver kinase B1 (LKB1) and its major downstream effector 5’ AMP-activated protein kinase (AMPK). This results in suppression of energy consuming pathways like lipogenesis and gluconeogenesis and activation of energy producing pathways like β-oxidation and ketone body synthesis. LKB1 is crucial for
metabolic homeostasis, as its loss in the liver results in hyperglycemia due to increased gluconeogenesis and upregulated lipogenesis. While LKB1 and AMPK are expressed uniformly across the liver lobule, processes they regulate in glucose and lipid metabolism are highly zonated along the portal to central vein axis of the lobule, a phenomenon known as metabolic zonation. Gluconeogenesis and β-oxidation predominate in periportal hepatocytes and lipogenesis and glucose uptake for glycolysis predominate in pericentral hepatocytes. However, it’s unclear whether LKB1 plays a role in maintaining hepatic metabolic zonation of glucose and lipid metabolism. To test this, we used a hepatocyte-specific LKB1 knockout mouse model (LKB1 KO) and a combination of confocal and intravital imaging to evaluate the spatial organization of glucose and lipid metabolism in the LKB1 KO liver. Here, we show that LKB1 KO mice lose the spatial zonation of lipid and glucose utilization and synthesis. Specifically, gluconeogenesis, β-oxidation and lipogenesis enzymes lost their restricted distribution on the porto-central axis in LKB1 KO mice. Concomitantly, we observed loss of lipid droplet zonation due to their periboral accumulation and reduced glucose uptake throughout the lobule in LKB1 KO mice. Interestingly, we observed that LKB1 KO mice lose weight despite adequate food intake, resulting in increased energy expenditure which indicates metabolic inefficiency. As mitochondria are the main ATP producers, we investigated their structure and observed marked changes in mitochondrial morphology across the lobule, possibly due to the imbalance of fission and fusion protein levels in the LKB1 KO mice. Current studies investigate mitochondrial function by measuring mitochondria calcium uptake in vivo. Overall, our results suggest that loss of LKB1 can lead to loss of zonation of glucose and lipid metabolism, and as a consequence may result in whole body energy imbalance.

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Docosahexaenoic Acid Differentially Modulates the Cell Cycle and Metabolism-Related Genes in Prostate Cells and Delays Tumor Progression in TRAMP Mice

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Docosahexaenoic acid (DHA) antiproliferative properties have been described in several tumors associated to cell metabolism regulation. This is of particular interest for prostate cancer (PCa), since most of available approaches target the androgenic pathway which often lead to reoccurrence. In the present study, we investigated the mechanisms underlying the antiproliferative potential of DHA on human prostate cells with distinct molecular contexts, particularly the cell cycle regulation and modulation of metabolism and androgen-regulated genes. In addition, we tested in vivo the omega-3 antitumor property. For this purpose, pre-malignant PNT1A (AR-FL+;PTEN+) and tumor 22rv1 (AR-FL+;AR-V7+;PTEN+) and PC3 (AR-; PTEN-) prostate cells were incubated with DHA at 100µM-48h. DHA reduced at least 26% cell number for all lineages due to S-phase decrease in AR-positive and G2/M arrest in AR-negative. Mitochondrial metabolic rate decreased in PNT1A (~38%) and increased in tumor cells (at least 40%) which was associated with ROS overproduction (1.6- PNT1A; 2.1- 22rv1; 2.2-fold PC3), lipid accumulation (3-fold PNT1A; 1.8 22rv1; 3.6 PC3) and mitochondria damage in all cell lines. Analyses by qRT-PCR array of 252 genes for nuclear receptors, androgen signaling and mitochondrial pathways showed that DHA affected each cell line in distinct pattern, but most of altered genes were involved with metabolism regulation, response to hormones, lipids and stress. The mitochondrial genes
were mainly affected in PNT1A with 52 downregulated whereas only Slc25a20 was upregulated in 22rv1 and Sh3glb1 downregulated in PC3, all of them related to organelle homeostasis, transport and mitochondrial organization. Also, the omega-3 led to mitochondrial network fragmentation and loss of the membrane potential in PNT1A in addition to decrease of basal oxygen consumption rate, which was not evident for tumor cells. AKT, AMPK and PTEN phosphorylation did not change in any cell line, but p-ERK1/2 increased (1.5-fold) in PNT1A. To validate the DHA antitumor potential, we provided DHA-enriched diet to TRAMP mice from the 8th week of age to euthanasia at 12th. Histopathological analysis of dorsolateral prostate based on estimation of lesions focus frequency showed that DHA decreased the high-grade PIN at 68% and carcinoma in situ at 63%. DHA intake also increased the areas of normal epithelial (2-fold) and reduced cell proliferation (65%) when compared to control group. In conclusion, DHA delayed PCa progression in vivo and our findings in vitro suggested that, regardless of androgenic or PTEN background, its antiproliferative effect is associated to mitochondria dysfunction, cell cycle impairment via a distinct gene regulation, but most of them related to metabolism and response to hormones.

The antiviral sirtuin 3 bridges protein acetylation to mitochondrial integrity and metabolism during human cytomegalovirus infection
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Regulation of mitochondrial structure and function is a central component of numerous viral infections as a virus means to modulate cellular metabolism and immune responses. During infection with human cytomegalovirus (HCMV), mitochondria undergo fragmentation and alterations in composition. Accumulating evidences have placed mitochondrial protein acetylation into the spotlight, given the HCMV-induced global elevation of the mitochondrial acetylome and the antiviral function of the mitochondrial deacetylase SIRT3. Here, we link SIRT3 enzymatic activity to its defense function and regulation of both mitochondrial structural integrity and metabolism during HCMV infection. We establish that SIRT3 deacetylase activity is necessary for suppressing virus production, and that SIRT3 maintains mitochondrial pH and membrane potential during infection. By defining the temporal dynamics of SIRT3-substrate interactions during infection, and overlaying acetylome and proteome information, we find altered SIRT3 associations with the mitochondrial fusion factor OPA1 and acetyl-CoA acyltransferase 2 (ACAA2), concomitant with changed acetylation levels. These alterations point to virus-induced changes in mitochondrial organization and fatty acid metabolism. Using mutagenesis, microscopy, and virology assays, we determine OPA1 modulates mitochondrial morphology of infected cells and inhibits HCMV production, depending on its K931 acetylation state, a site regulated by SIRT3. Furthermore, modulation of SIRT3 protein levels or enzymatic activity is sufficient for regulating mitochondrial filamentous structure. Lastly, we establish a virus restriction function for ACAA2, an enzyme regulating fatty acid beta-oxidation. Altogether, we highlight SIRT3 activity as a regulatory hub for mitochondrial acetylation, integrity, metabolism during HCMV infection, and point to global acetylation as a reflection of mitochondrial health.
Global changes in HepG2 metabolism in response to galactose treatment

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Tumor cell proliferation requires sufficient metabolic flux through the pentose phosphate pathway to meet the demand for biosynthetic precursors and to increase protection against oxidative stress which in turn requires an upregulation of substrate flow through glycolysis. This metabolic poise is often coupled with a shift in ATP production from mitochondrial OXPHOS to substrate-level phosphorylation. Despite major advances that were facilitated by using tumor-derived cell lines in research areas spanning from membrane to cytoskeletal biology, this distorted metabolic profile limits their impact as a model in physiology and toxicology. Substitution of glucose with galactose in the cell culture medium has been demonstrated to shift ATP production from substrate-level phosphorylation to mitochondrial OXPHOS. This increase in oxygen utilization is coupled to a global metabolic reorganization with potential impacts on macromolecule biosynthesis and cellular redox homeostasis, but a comprehensive analysis on the effects of sugar substitution in tumor-derived cells is still missing. To address this gap in knowledge we performed transcriptomic and metabolomic analyses on human hepatocellular carcinoma (HepG2) cells adapted to either glucose or galactose as the aldohexose source. We observed a shift towards oxidative metabolism in all primary metabolic pathways at both transcriptomic and metabolomic levels. We also observed a decrease in nicotinamide dinucleotide (NAD(P)) levels and subcellular NAD+ to NADH ratios in cells cultured with galactose compared to glucose control cells. Our results suggest that galactose reduces both glycolytic and biosynthetic flux and restores a metabolic poise in HepG2 cells that closely reflects the metabolic state observed in primary hepatocytes.

Effects of androgenic stimulation on mitochondrial respiration and organelle distribution in prostatic epithelial cells

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Puberty is an important stage of prostate development, having as one of its main events the beginning of secretory activity by specialized epithelial cells. Among the secretion components, the high concentration of citrate ion stands out, suggesting a blockage of aconitase, the enzyme responsible for citrate conversion in the citric acid cycle. It is known that during the first postnatal weeks the physiological states of the prostate are tightly regulated by androgens, with an expressive increase in testosterone concentration at puberty. However, morphofunctional aspects of epithelial cell mitochondria in variable conditions of androgenic stimulation are poorly elucidated. Therefore, we aimed to verify the organization of the mitochondrial network of epithelial cells and mitochondrial oxygen consumption in ventral prostate (VP) biopsies of rats belonging to two groups in physiologically distinct phases from normal prostate development: pre pubertal and post pubertal; opposed to a third
one induced to androgenic deprivation: post pubertal killed on day 3 after surgical castration. Airyscan microscopy images obtained after staining with MitoTracker® Red CMXRos + HOECHST 33342 revealed a disparity in the distribution of mitochondrial content throughout the VP epithelial ducts (proximal, medial and distal regions, according to their position regarding the urethra). The absence of androgenic stimulation seems to impact the mitochondrial network promoting the appearance of mitochondrial clusters, despite similar aggregates are also present in proximal regions of pre pubertal rats not subjected to castration. Results obtained by high-resolution respirometry indicated an upward trend in the average respiratory rates in the post pubertal group when compared to the prepubertal animals: ADP (26%), oligomycin (20%) and FCCP (24%). Successively, castration has contributed to a significant decrease in average respiratory rates: ADP (45%), oligomycin (47%) and FCCP (28%). These observations suggest an association between androgenic stimulation and mitochondria in the rat VP, with androgenic deprivation impacting mitochondrial oxygen consumption and promoting morphological changes with the appearance of mitochondrial aggregates. Financial support: CNPq, FAPESP and CAPES. INFABiC is co-funded by FAPESP (2014/50938-8) and CNPq (465699/2014-6). FBST is supported by a fellowship from CNPq (140512/2020-9).

Molecular Mechanisms of Morphogenesis

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Cleavage of Dally-like protein by Matrix Metalloprotease 2 inhibits Wg/Wnt signaling by sequestration of Wnts
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Extracellular distribution of secreted Wingless (Wg)/Wnt1 from source cells to recipient cells is crucial for proper tissue development and patterning. The recipient cells respond to secreted Wg in a dose-dependent manner, and thus the level of Wg available for signaling in the extracellular space must be tightly regulated. Dally-like protein (Dlp) is a cell-surface glypican that binds to secreted Wg and regulates its distribution in the extracellular space by restricting paracrine Wg availability and promoting long-range Wg availability, fine tuning paracrine and long-range Wg signaling. In addition to regulating Wg ligand, Dlp also restricts paracrine availability of other Wnts, Wnt2, Wnt4, and Wnt6 in Drosophila gerarium, a tissue where oogenesis initiates. Genetic studies suggest that the cleavage of Dlp by Matrix Metalloprotease 2 (Mmp2) attenuates long-range distribution of Wg from source cells to recipient cells in the gerarium, and Mmp2 cleaves Dlp within the N’ terminal subunit in cell-culture experiments. However, how the cleavage of Dlp by Mmp2 attenuates long-range Wg signaling is not clear. We find that Mmp2-mediated cleavage of Dlp results in increased co-immunoprecipitation of Wg/Wnt2/Wnt4/Wnt6 ligands with cleaved Dlp. These findings suggest that the cleavage of Dlp by Mmp2 alters Dlp-Wnt interaction to modulate paracrine and long-range Wg/Wnt availability and signaling. We are currently testing the hypothesis that the Wg/Wnts sequestered by cleaved Dlp are endocytosed and degraded to attenuate Wg/Wnt signaling. Thus, our study sheds light on how metalloproteases and glypicans regulate Wg/Wnt signaling ranges.
Clues of multichambered heart evolution are revealed through analysis of zebrafish hearts with adaptive remodeling of the sinus venosus

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The mechanisms underlying the evolutionary transition from the single chambered heart observed in ancestral chordates to the two-chambered pump exhibited by basal vertebrates remain a mystery. Here, we use zebrafish mutants harboring a novel hypomorphic nr2f1a allele (nr2f1aacro) that can survive to adulthood despite embryonic heart defects. Adult nr2f1aacro hearts exhibit enlarged ventricles and overtly malformed “atria.” However, histological and marker analysis showed that nr2f1aacro mutants essentially lack a true atrium. Instead, it has been replaced by a chamber with thickened, collagenous tissue that RNA-seq indicated is the sinus venosus (SV), suggesting the SV has undergone adaptive remodeling in the absence of an atrium. Surprisingly, our transcriptomic analysis of all the cardiac chambers coupled with subsequent lineage tracing and reporter analyses of smooth muscle and neural crest also highlighted previously unknown molecular and cellular similarity between the wild-type (WT) bulbus arteriosus (BA) and SV. Furthermore, principal component analysis indicated that the transcriptional signature of the nr2f1aacro mutant SV becomes even more similar to the thick-walled WT BA. We posited that aberrant blood flow in nr2f1aacro hearts may cause a pressure increase within the SV that elicits the adaptive remodeling. Indeed, echocardiography showed retrograde blood flow in mutant hearts and that prolonged treatment with a vasodilator mitigated this response. Altogether, we find nr2f1aacro hearts come to resemble a single-chambered pump, reminiscent of the bidirectional hearts found in urochordates, except they are flanked by two elastic vessels. Our examination of mechanisms promoting adaptive remodeling of the SV in response to changes in hemodynamic forces highlights unexpected similarity between arterial and venous poles of the zebrafish heart, which hints at anatomical characteristics of the basal heart during the evolution to a multi-chamber heart.

A wholistic approach to investigating the epithelial to mesenchymal transition (EMT) in human induced pluripotent stem cells (hiPSC)

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The Allen Institute for Cell Science is developing a state space of structural signatures to understand the principles by which human induced pluripotent stem cells (hiPSC) traverse the cell cycle and differentiate to cardiomyocytes. Towards this goal, we have produced hiPSC lines in which protein markers of particular cell structures or organelles have been endogenously tagged with mEGFP (the Allen Cell Collection), and we have developed standardized 3D imaging platforms and computational tools for these cells (www.allencell.org). Previously, we have combined the quantification of intracellular structural organization with transcript abundance in the same cell (via scRNA-seq informed RNA FISH) to reveal different states in cardiomyocyte organization (Gerbin KA et al. Cell states beyond transcriptomics: integrating structural organization and gene expression in hiPSC-derived cardiomyocytes 2020 bioRxiv doi: 10.1101/2020.05.26.081083). We are now applying a similar multi-modal and multi-scale approach to investigate a state change that happens much earlier in the hiPSC to cardiomyocyte differentiation process, that of the epithelial to mesenchymal transition (EMT). EMT is a
process that occurs normally, e.g. during development and in wound healing, as well as pathologically, as in cancer metastasis. Perhaps due to its role in many contexts and across several different cell types, a wholistic view of EMT is lacking. Here, we show how long-term time-lapse imaging of appropriate Allen Cell Collection lines through the duration of EMT, combined with EMT-related immunolabeling, scRNA-seq, and RNA FISH data from cells collected at specific timepoints during the process can inform an integrated view of the state change(s) during EMT. We believe this approach might also serve as a template for the study of EMT in other cell systems and contexts.

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**Combination of soluble factors and biomaterial scaffolds enhance human adipose-derived stem/stromal cell myogenesis**

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Volumetric muscle loss (VML) is characterized by muscle injury that does not regenerate naturally and can occur due to traumatic injuries, surgical procedures, or abnormal muscle conditions. The current treatments for VML are limited by donor tissue availability, the invasiveness of procedures, and an increase in scar tissue formation and advanced bracing. Therefore, there is a growing need to find a solution to this debilitating condition. Producing 3D tissue scaffolds that direct skeletal muscle differentiation has the potential to revolutionize the way in which VML is treated. Human adipose-derived stem cells (hASCs) offer promise in cell-based regenerative therapies to treat muscle damage due to their ability to self-renew, their multipotent properties, their ability to be readily harvested from patients through minimally invasive lipoaspirates, and their relative abundance. The clinical potential of hASCs for treating VML is currently limited due to a lack of universal culture conditions and low myogenic differentiation efficiency. The challenge with increasing myogenic differentiation lies in creating an environment that mimics the natural extracellular matrix (ECM) found in skeletal muscle tissue and promotes the reliable differentiation of stem cell populations towards a myogenic lineage. In this work two different media recipes reported in literature, three ECM proteins, and a poly (ethylene glycol) (PEGDMA) hydrogel with a physiologically relevant elasticity were evaluated to determine how the extracellular chemical and physical environment work together to enhance differentiation of hASCs towards a myogenic lineage. Results suggest that hASCs cultured in media containing horse serum and hydrocortisone, with 5-azacytidine on collagen type 1 coated PEDGMA hydrogel scaffolds increased MYOD expression at a faster rate when compared to all other combinations of media and ECM proteins. Myogenic differentiation was confirmed through RT-PCR of early, middle, and late muscle markers in conjunction with immunofluorescence of MYOSIN and MYOD. The results identify a combination of biochemical factors that promotes myogenesis of hASCs, laying the groundwork for creating an 3D scaffold to efficiently direct myogenic differentiation of adult stem cells for the repair and replacement of muscle tissue for clinical applications.
**P582**

Craniofacial cartilage organoids from human embryonic stem cells via a neural crest cell intermediate

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During neurulation, neural crest cells (NCCs) are specified at the neural plate border, located between the neural plate and non-neural ectoderm. NCCs delaminate and migrate throughout the developing embryo, differentiating into several lineages of cells including neurons and glia of the peripheral nervous system, secretory cells, melanocytes, and the majority of craniofacial cartilage and bone. Here we describe a new protocol for NCSC differentiation from both human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs). From differentiated NCSCs we have further derived craniofacial cartilage organoids. Cartilage organoids self-organized in culture dishes and expressed several collagen isoforms and collagen receptors. Organoids also expressed markers indicative of neural crest lineage and were continuously cultured for one year, reaching up to one centimeter in diameter. Histological staining of cartilage organoids revealed tissue architecture typical of hyaline cartilage. Organoids were composed of rounded aggregates of glassy, gray matrix that contained scattered small nuclei in lacunae. Mass spectrometry analysis of cells at different stages of differentiation indicated that growth factors secreted by organoids may contribute to the formation of an autocrine loop that promotes chondrocyte differentiation. Organoids treated with combinations of specific ligands identified from mass spectrometry exhibited accelerated growth, supporting the hypothesis that a positive feedback loop may promote craniofacial cartilage differentiation. These results provide further insight into the mechanisms driving neural crest differentiation into craniofacial cartilage, which is important for the understanding of normal human development and diseases that originate from neural crest tissues.

**P583**

Sbf and Rab21 control autophagic flux to modulate drosophila intestinal stem cell fate.

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The intestinal epithelium faces many stresses caused by food digestion or its proximity to microorganisms among others, explaining its rapid renewal. Mechanisms, such as autophagy, can be induced by immune or environmental stressors to control gut homeostasis. Dysfunctional autophagy has been associated with inflammatory bowel pathologies. However, its specific roles in intestinal stem cell remain poorly investigated. Since this intracellular catabolic mechanism also plays a role in the maintenance of stemness or the differentiation of dermal and hematopoietic cells, we wondered if it may contribute to the renewal of the intestinal epithelium. To investigate this question, two new autophagy regulators were studied: the small GTPase Rab21 and the myotubularin-related protein Sbf. During starvation, Sbf activates Rab21 to induce the endosomal sorting of the lysosomal SNARE Vamp7, required for autophagosome-lysosome fusion. Moreover, Rab21 was recently identified in a genome-wide screen as a potential intestinal stem cell regulator. Taken together, we hypothesized that dysfunctional autophagy caused by the depletion of either Sbf or Rab21 would disturb drosophila intestinal stem cell fate. As in mammal, drosophila intestinal epithelium renews every 5-7 days. Intestinal stem cells (ISCs) divide into specific progenitors, either pre-enterocytes or pre-
enteroendocrines that differentiate into nutrient-absorbent enterocytes or hormone-secretive
enteroendocrine cells respectively. To inhibit autophagy, we used the Gal4-UAST system to express
interfering RNA against Sbf, Rab21 or Vamp7 (positive-control) specifically in adult ISCs and/or
progenitor cells, along with GFP to allow the quantification by confocal microscopy of depleted cells.
Our results showed that autophagy inhibition specifically in ISCs or in pre-enterocytes does not affect
their number but surprisingly increases the proportion of enteroendocrine cells. Using a lineage tracing
method, we confirmed that the depletion of either Sbf, Rab21 or Vamp7 in ISCs and progenitor cells
increased the number of newly formed-enteroendocrine cells as well as pre-enteroendocrine cells,
without disturbing the ratio between ISCs and progenitors. Thus, our data suggest that autophagy
regulates the differentiation of intestinal cells into enteroendocrine cells in drosophila.

Neuronal Degeneration and Regeneration

P584

α-Synuclein induces coflin pathology: implications for Parkinson’s disease dementia
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Dementia is recognized as a major complication in Parkinson’s disease (PD), neglected in the past by the
typical PD motor symptoms. PD-dementia appears to be associated with the accumulation of alpha-
Synuclein (αSyn) in the hippocampus, occurring in patients presenting multiplication of the αSyn
encoding gene SNCA. In this respect, we investigated the effect of elevated levels of αSyn, either by
protein overexpression or by exogenous addition of pre-formed fibrils of αSyn (PFFs), on primary
cultures of hippocampal neurons and hippocampal slices, and we observed the formation of coflin-actin
rods. These structures are formed upon localized coflin hyperactivation by dephosphorylation, leading
to its co-assembly in a 1:1 filament complex with actin, which bundle into rods under conditions of
oxidative stress. Cofilin-actin rods have been reported in human Alzheimer’s disease (AD) and in mouse
models of AD, where they are induced by Amyloid-β oligomers via a cellular prion protein (PrP⁰)-
dependent pathway in hippocampal neurons, promoting alterations in axonal transport and cause
synaptic impairment underlying cognitive dysfunction. In our settings, αSyn-induced coflin pathology
affected dendritic spine number and morphology and occurred through a mechanism involving PrP⁰ and
the NADPH oxidase pathways. Importantly, we found hippocampal coflin pathology beginning at the
same age as cognitive dysfunction is observed in a mouse model of human SNCA triplication. We also
have preliminary data showing rod formation in hippocampal sections from PD patients where cognitive
impairment was reported. Currently, we are evaluating in vivo the consequences of coflin pathology, as
well as the effect of its blockage using rod inhibitors, on synaptic dysfunction and cognitive impairment.
This work supports the innovative hypothesis that coflin pathology, occurring specifically in brain
regions related with cognition, underlies synaptic impairment and cognitive dysfunction in PD.
**P585**

**A cell-autonomous role for Nox-derived ROS in neurite regeneration**

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Reactive oxygen species (ROS) at high concentrations are well known for their damaging effects to cell components and contribution toward aging, cancer, and neurodegeneration. However, they can also be important signaling agents at physiological concentrations. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) is a major source of cellular ROS and has been implicated in nervous system development including neurogenesis, neuronal polarity, and axonal growth. Recently, Nox has been shown to regulate axonal regeneration of sensory neurons following injury in a non-cell autonomous manner. However, whether Nox also has a cell-autonomous role in axonal regeneration is unclear thus far. Previously we found that Nox2 is expressed in growth cones of cultured *Aplysia* bag cell neurons. Here, we tested a role for Nox-derived ROS in neurite regeneration following mechanical injury of *Aplysia* neurons. Hydrogen peroxide (H₂O₂) levels in neuronal growth cones were determined with different H₂O₂-sensing dyes, including the novel dye p-bispinacolaboron-5'-phenylpyridylthiazole (Bpin-PPT). We found that H₂O₂ levels were increased in *Aplysia* bag cell neurites following injury. Furthermore, low doses of the Nox inhibitor Celastrol (CST) enhanced the rate of neurite regeneration, whereas high doses of CST had opposite effects. These findings suggest that the levels of neuronal Nox-derived ROS are critical for neurite regeneration after injury.

**P586**

**Identification of extrinsic cues promoting target-selective axon regeneration**

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Regenerating axons in the peripheral nervous system must extend over long distances to reconnect with their original synaptic targets for functional recovery. However, re-establishing a complex trajectory that includes navigating multiple choice points and then selecting the appropriate target long after this circuitry was established during development, represents a unique challenge. To visualize regenerating axons as they navigate stepwise choice points, we established the larval zebrafish pectoral fin, equivalent to tetrapod forelimbs, as a vertebrate model system in which to study this process. Each pectoral fin is innervated by four motor nerves containing dozens of axons that branch to stereotypically-innervate specific regions of two muscle layers. Using a laser, we transect the fin motor nerves and monitor axon regeneration in real time. We have characterized the stepwise choices faced by regenerating axons including sorting at the plexus to select the correct muscle layer, selectively fasciculating with the appropriate axonal partners to grow into the fin, and then defasciculating to reinnervate their original individual muscle fibers. By labeling single axons, we observe robust, specific, and functional regeneration of motor axons back to their original domains within 48 hours post injury, indicating the existence of local cues within the fin to guide selective reinnervation. To identify extrinsic injury-dependent guidance cues in the pectoral fin, we have employed an RNAseq approach. We present results from this RNAseq analysis from denervated fins at timepoints that precede important axon guidance decisions including choosing a muscle layer, sorting at the plexus, and specific target selection. We predict that changes in gene expression may reflect regional cues important for axon
growth and guidance and are testing mutants in genes that are upregulated after injury to determine their functional role in axon guidance. Funding: NINDS NRSA F32NS103219

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**Ca\textsuperscript{2+}/calcineurin mediated release of exosomal miR-23a has a role in astrocyte dysfunction and neurodegeneration in Parkinson's disease model**

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**Rationale:** Parkinson’s disease (PD) is the second most prevalent neurodegenerative disorder affecting mainly the midbrain dopaminergic neurons (mDN). Astrocyte dysfunction is well implicated in a number of neurodegenerative diseases, including PD. Now, Ca\textsuperscript{2+}/Calcineurin signaling is reported to induce astrocyte dysfunction in Alzheimer’s disease (1) but very little is known in context to PD. So, our objective was to study the role of Ca\textsuperscript{2+}/Calcineurin signaling in astrocyte-neuron cross-talk during PD which is unreported so far. **Methods:** 1. Mammalian cell culture. 2. Rotenone treatment. 3. MTT assay. 4. Immunoblotting. 5. qRT-PCR. 6. Exosome isolation. 7. Intracellular Ca\textsuperscript{2+} measurement. 8. Transfection. 9. 3'UTR cloning. **Results:** As a cellular model of PD, we chose the human astrocytic 1321N1 cell line and treated the cells with varying doses of Rotenone, eventually choosing a dose which showed 40% death in 24 hrs. A simultaneous activation of the Ca\textsuperscript{2+}-dependent protein phosphatase, Calcineurin and an intracellular surge in the Ca\textsuperscript{2+} levels were also observed at this dose. Now, miR-23a is an anti-apoptotic miRNA and Calcineurin/NFAT-mediated upregulation of miR-23a has been reported in cardiac hypertrophy (2). Surprisingly, in our study, Rotenone treatment caused a Calcineurin-mediated decrease of intracellular miR-23a levels in astrocytes. Now this decrease, we observed, was due to a concomitant release of miR-23a via exosomes. So the next question we asked was whether the astrocytes release this anti-apoptotic miR-23a via exosomes to protect the neurons. To answer this query, we treated neurons with Rotenone and found that the pro-apoptotic protein PUMA was upregulated in those neurons. However, the neurons showed no change in the miR-23a levels upon Rotenone treatment. Interestingly, overexpression of miR-23a showed attenuation of the Rotenone mediated death in neurons. Since PUMA is a predicted target of miR-23a, we have cloned the 3'UTR of PUMA to check by luciferase assay if miR-23a can directly bind to it, in our study. **Conclusion:** Our results so far, suggest a novel mechanism by which astrocytes cause Ca\textsuperscript{2+}/Calcineurin mediated release of miR-23a through exosomes which, in turn, play a probable role in protection of Rotenone induced death of mDN in PD. **Acknowledgement:** We thank Prof. Sharmistha Banerjee (Hyderabad University) for providing the 1321N1 cell line and to CSIR, Gol, for funding the project. The authors declare no conflict of interest. **References:** 1. Sompol P. et al. Calcineurin/NFAT Signaling in Activated Astrocytes Drives Network Hyperexcitability in Aβ-Bearing Mice. J Neurosci. 2017 Jun 21;37(25):6132-6148. 2. Lin Z. et al. miR-23a functions downstream of NFATc3 to regulate cardiac hypertrophy. Proc Natl Acad Sci U S A. 2009 Jul 21;106(29):12103-8.

P588

**The interaction of tau protein with filamin A contributes to its accumulation in neuronal cells**

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In Alzheimer disease (AD), tau, an axonal microtubule associated protein, becomes hyperphosphorylated, accumulates and aggregates in the somatodendritic compartment. The
accumulation of hyperphosphorylated and aggregated tau is also seen in other neurodegenerative diseases called tauopathies such as frontotemporal dementias (FTDs) linked to tau mutations. This tau accumulation correlates with the onset of cognitive impairment in patients. The mechanisms leading to tau accumulation remain poorly characterized. Our hypothesis is that hyperphosphorylated tau interacts with a protein complex that reduces its degradation by the proteasome and/or autophagy. Studies reported the presence of filamin A (FLNA), an actin binding protein, in tau aggregates. Interestingly, it was observed that the interaction of FLNA with a protein can reduce its degradation. Our preliminary results obtained from a small number of patients revealed a tendency for FLNA to be increased in AD brain. In the neuroblastoma cell line (N2a), we demonstrated that overexpression of FLNA resulted in an intracellular accumulation of tau wild-type and tau mutants (P301L, R406W and V337M) linked to FTDs. Our results also revealed that upon FLNA overexpression, tau phosphorylation is increased at certain sites and decreased at other sites. To examine whether phosphorylation was a determinant factor in tau accumulation, we used a mutant either mimicking phosphorylation of tau or a mutant not phosphorylatable at 12 sites known to be phosphorylated in AD (E12 and A12 respectively). The intracellular accumulation of tau upon FLNA overexpression seems to be independent of tau phosphorylation state. Our preliminary results using GFP-Trap revealed that the interaction between FLNA and tau is mediated by a tau domain located in 214-441 amino acids. All together, our results demonstrate that FLNA interaction with tau could contribute to its accumulation in AD and therefore preventing this interaction could reduce tau accumulation and its detrimental effects on neurons.

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*Achillea millefolium* flower petroleum ether extracts mediate neuroprotection via AKT and mTOR pathway

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Parkinson’s disease (PD) is a highly prevalent neurodegenerative disorder that affects over 6 million people with the majority of the affected population being over the age of 65. PD is considered to be a chronic and progressive disorder that affects the dopaminergic neurons. It is characterized by the loss of degeneration of these neurons in the substantia nigra region of the brain which further negatively impacts the ability of dopamine signaling. Much research has assisted to elucidate the pathogenesis of PD, however the precise etiology of this disease still remains unknown. Research has shown that the mTORc and AKT signal exerts cytoprotective effects in cellular models of many diseases, including Parkinson’s disease. This study aimed to investigate the neuroprotective effects of activated AKT signal by mTOR on SH-SY5Y cells treated with 6-hydroxydopamine (6-OHDA).

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Neuroprotective Mechanisms of *Achillea millefolium* Against Toxin-treated SHSY5Y Cells


Parkinson's Disease (PD) is an age-related neurodegenerative disorder that affects the dopamine-producing neurons of the brain. There are currently over 5 million people with PD worldwide. PD symptoms include a loss of motor skills, an increase in tremors, rigidity, and slurred speech. Previous
research on PD has identified many targets and pathways that are affected by PD; however, the full etiology is unknown and provides a key area of research to explore. Current treatments for PD include levodopa and carbidopa; however, with continuous use, side effects such as dyskinesia and hallucination occur. Because of this, it is paramount to discover a drug for PD that can decrease PD associated symptoms and slow down the rate of degeneration of neurons without the subsequent increase in side effects. We propose the use of phytochemicals derived from the plant Achillea millefolium, a known anti-inflammatory agent, to alleviate PD associated symptoms, and to protect neurons from further damage by reducing oxidative stress linked with PD progression. Bioassay-guided fractionation is used to isolate and evaluate compounds from A. millefolium for neuroprotective effects. MS and NMR will be used to determine the identity of compounds that give the desired effects. These include; reduction of ROS, increase in antioxidant enzyme activity, regulation of apoptotic signals, regulation of neurotransmitters, increase in dopamine production, decrease in alpha-synuclein protein aggregation, lipid peroxidation, and inflammation. By evaluating the neuroprotective effects of our candidate compounds, we will be able to identify a promising therapeutic for PD.

Other Myosins

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Fast and Directionally Switchable Engineered Myosins Enable Optical Localization of Motor-Driven Cargos In Live Cells

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Spatiotemporal control of cytoskeletal transport can provide new possibilities for dissecting cellular processes. Optogenetic approaches have been used for both controlled recruitment of motors to cellular cargos [1] and direct modulation of motor speed and direction [2]. Here we report optimized and diversified engineered myosin motors with velocities and directionality that can be optically controlled using dynamic changes in lever arm geometry. Previous designs for light-activated gearshifting [2] were non-processive, and suffered from either low velocities (< 10 nm/s) or modest degrees of velocity modulation (~15%) in response to light. These limitations preclude many applications in cell biology, devices, and reconstituted systems. We have now engineered (i) non-processive myosin motors that combine large optical modulation depths with high velocities and (ii) processive myosin motors with optically controllable directionality. We have characterized a series of optimized constructs using in vitro motility assays of propelled actin filaments, single-molecule tracking of processive complexes, and live cell imaging. For processive myosins, we demonstrate cellular localization at filopodial tips under optical stimulation, in fibroblasts and cultured neurons. Finally, we demonstrate

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The small EF-hand protein CALML4 functions as a critical myosin light chain within the intermicrovillar adhesion complex

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Specialized transporting and sensory epithelial cells employ homologous protocadherin-based adhesion complexes to remodel their apical membrane protrusions into organized functional arrays. Within the intestine, the nutrient-transporting enterocytes utilize the intermicrovillar adhesion complex (IMAC) to assemble their apical microvilli into an ordered brush border. The IMAC bears remarkable homology to the Usher complex, whose disruption results in the sensory disorder type 1 Usher syndrome (USH1). However, the entire complement of proteins that comprise both the IMAC and Usher complex are not yet fully elucidated. Using a protein isolation strategy to recover the IMAC, we have identified the small EF-hand protein calmodulin-like protein 4 (CALML4) as an IMAC component. Consistent with this finding, we show that CALML4 exhibits marked enrichment at the distal tips of enterocyte microvilli, the site of IMAC function, and is a direct binding partner of the IMAC component myosin-7b. Moreover, distal tip enrichment of CALML4 is strictly dependent upon its association with myosin-7b, with CALML4 acting as a light chain for this myosin. We further show that genetic disruption of CALML4 within enterocytes results in brush border assembly defects that mirror the loss of other IMAC components and that CALML4 can also associate with the Usher complex component myosin-7a. Our study further defines the molecular composition and protein-protein interaction network of the IMAC and Usher complex and may also shed light on the etiology of the sensory disorder USH1H.

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Cryo-em structure of the shutdown state of smooth muscle myosin

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The human myosin superfamily is made up of 12 different myosin classes. Class 2 is the largest with 13 genes. Of these 9 are found in striated muscle and the remaining 4 in non-muscle cells and smooth muscle. Class 2 myosins cycle between an extended conformation (6S) whereby they assemble into filaments and are active, and a shutdown conformation, in which the two heads (comprising the motor domain and lever) interact with the proximal region of the coiled coil tail to form the so-called ‘interacting heads’ motif. In non-muscle and smooth muscle myosin the distal part of the coiled-coil tail further interacts with the myosin heads enabling it to form the shutdown 10S structure. Phosphorylation of the regulatory light chain (RLC) disrupts the 10S structure activating the myosin.
Using cryoEM, we solved the structure of the 10S state of smooth muscle myosin, with a resolution of ~9Å in the region of the myosin heads. The structure reveals the details of the interaction between the coiled-coil tail and the myosin heads, and how RLC phosphorylation destabilises the shutdown state.

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**Characterization of deafness mutation in Myo3A reveals actin protrusion elongation mechanism**

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Class III myosins are actin-based motors proposed to transport cargo to the stereocilia tips in inner ear hair cells and/or to participate in stereocilia length regulation, especially during development. MYO3A mutations are associated with DFNB30, an autosomal recessive form of delayed onset deafness. Recently, we characterized a dominant mutation, L697W, in the MYO3A motor domain. Cell biological studies demonstrated that the L697W mutant localizes to actin protrusions more efficiently than WT MYO3A in the presence of ESPN1. We found that the L697W mutant slows in vitro motility 2-fold while it decreases ATPase more than 4-fold and increases actin affinity 2-fold. We also found that the ADP release rate constant was reduced 2-fold in L697W, which is consistent with its slower in vitro motility. We also found the mutant did not change the rate of ATP binding or ATP-induced dissociation from actin. Our single turnover data demonstrates that MYO3A has a post-hydrolysis turnover rate that is similar to the ADP release rate constants in both WT and L697W, suggesting that the ATP hydrolysis step may be rate-limiting. Thus, we hypothesize that there may be little change in the MYO3A motor duty ratio since the slowed ADP release and ATP hydrolysis have opposing effects on the duty ratio. Fluorescence recovery after photobleaching (FRAP) was used to examine the residence time at the filopodia tips of COS7 cells. We found that the mutation has no impact on the MYO3A turnover at protrusion tips or the average intensity at the tips. We utilized total internal reflection microscopy (TIRF) to demonstrate a significant reduction in the average filopodia length and filopodia extension rate in L697W MYO3A containing filopodia compared to WT. Overall, our work highlights that the motor properties MYO3A are crucial for allowing it to facilitate elongation and regulate actin protrusion lengths.

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**Unc-82/nuak kinase is required in a myosin-isoform-specific manner for myosin a to assemble and function in the thick filament arms of *caenorhabditis elegans* striated muscle**

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The assembly and maintenance of the complex cytoskeleton of striated muscle involve mechanisms whose elucidation will provide insights into both human myopathies and the proteostasis pathways that operate in other cell types. Striated muscles contain highly organized myosin-containing thick filaments, which are anchored to M-lines, and intercalated actin-containing thin filaments, which are anchored to homologous structures called dense bodies in worms and Z-disks in mammals. Mutations affecting the *C. elegans* M-line protein UNC-82, which is orthologous to mammalian kinases ARK5/NUAK1 and SNARK/NUAK2, result in abnormal thick filaments. Previous studies showed that UNC-82 is required for
proper assembly of paramyosin, a coiled-coil protein that forms the core upon which myosin assembles, and which is homologous to the filament-forming rod domain of myosin. In this study we demonstrate that UNC-82 kinase activity is also required for proper assembly and function of myosin A, but not myosin B, in the filament arms. Myosins A and B are located within different regions of the thick filament. Myosin A is essential for initiating assembly at the filament center and when over expressed can replace myosin B in the filament arms. Using transgenic double mutants, we show that myosin A requires UNC-82 kinase activity to form functional filament arms. Evidence that myosin B does not require this activity are the reciprocal effects of altering myosin B levels in a kinase-impaired unc-82 mutant: over expression of myosin B improves muscle structure and function whereas loss of myosin B results in severe, early-onset defects in paramyosin and myosin A pattern, prior to onset of contraction. Using chimeric myosins, we mapped the region of myosin A that requires UNC-82 kinase activity to a 531-amino-acid region within the rod. A physical association between myosin A and UNC-82 is suggested by colocalization in normal and mutant muscle, and by UNC-82::GFP labeling of ectopic myosin A aggregates but not ectopic thin filaments. The inability of increased myosin B to suppress an unc-82 null mutant suggests a kinase-activity-independent role for the large region C-terminal to the UNC-82 kinase domain. We hypothesize that UNC-82 is a myosin-A-specific assemblase or chaperone that promotes incorporation of myosin A during elongation of the filament arms.

Disease-associated MYO1E mutations have differential effects on myosin 1e localization and dynamics and clathrin-dependent endocytosis

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Mutations in the MYO1E gene, encoding Myosin 1e (Myo1e), are associated with steroid-resistant nephrotic syndrome (SRNS), a progressive kidney disease that can lead to kidney failure. Myo1e is a non-muscle myosin that is enriched in the renal glomerular epithelial cells (podocytes) and is involved in endocytosis, phagocytosis, and cell motility and adhesion in a variety of cells. We set out to determine whether novel MYO1E mutations found in SRNS patients using exome sequencing (Sadowski et al., JASN, 2015, 26(6): 1279-89) affect Myo1e functions, with the goal of differentiating between pathogenic and neutral variants. EGFP-tagged human Myo1e constructs containing selected mutations were delivered into Myo1e knockout mouse podocytes using adeno viral vectors. Both Myo1e-T119I and D388H mutants were expressed as full-length proteins. However, Myo1e-T119I was diffusely localized in the cytosol and, unlike wild-type Myo1e, it was not enriched at the cell-cell contacts or clathrin-coated vesicles in podocytes. Analysis of clathrin-dependent endocytosis using live-cell imaging by Total Internal Reflection Fluorescence Microscopy (TIRFM) revealed that the total density (puncta per unit area) of clathrin-coated vesicles and the peak intensity of Myo1e co-localized clathrin-coated vesicles were reduced in Myo1e-T119I expressing podocytes, which could indicate a shorter vesicle lifetimes or a decrease in endocytic vesicle formation. In contrast, Myo1e-D388H was similar to the wild-type myosin with regard to cell-cell contact and clathrin-coated vesicle localization. Surprisingly, the rate of protein exchange of Myo1e-D388H at cell-cell contacts, as measured by Fluorescence Recovery After Photobleaching (FRAP), was decreased, and the total density of clathrin-coated vesicles and peak
intensity of the Myo1e co-localized clathrin-coated vesicles was increased. Overall, our findings suggest that the two MYO1E motor domain mutations, T119I and D388H, likely have opposing effects on Myo1e motor activity and actin binding, with the T119I mutation decreasing myosin-actin interactions and the D388H mutation prolonging myosin-actin binding. Our analysis shows that these mutations are likely to be pathogenic and reveals the possible mechanisms leading to the disruption of kidney filtration in patients expressing mutant Myo1e.

Physical Approaches to Cell Biology 2

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Morphology of prothymosin α and linker histone h1 coacervates at various pH
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The interaction of oppositely charged polyelectrolytes can be accompanied by spontaneous liquid-liquid phase separation of the system. One phase is enriched with a polymer (coacervate phase), and the other is diluted. It is believed that complex coacervation of intrinsically disordered proteins may be one of the mechanisms of the membraneless organelles formation. The aim of this work was to determine the conditions under which the interaction of intrinsically disordered proteins, prothymosin α (ProTa) and linker histone H1 (H1), leads to the liquid coacervates formation in vitro. These proteins bear opposite charge at physiological pH. Using Rayleigh scattering method we showed that mixing of 25 μM ProTa with 46 μM H1 is followed by disturbance of the solution homogeneity at 4.0 < pH < 9.0. At such conditions the total positive charge of H1 is substantially neutralized by the negative charge of ProTa. The positive to negative charge ratio does not exceed 5:1. It is accepted, that such charge compensation is a required factor to guide protein assembly into spherical condensates [1]. Examination of turbid solution using differential interference-contrast microscopy showed that immediately after mixing ProTa and H1 form small spherical droplets. These droplets fuse with each other, confirming its liquid nature. However, no more than in two hours, the droplets lose their ability to fuse transforming to non-dynamic aggregates. These aggregates retain spherical structure even at ultrastructure (<200 nm) level. The obtained results suggest that liquid droplets assembling upon the ProTa and H1 interaction are an intermediates on the pathway of the stable non-dynamic aggregates formation. This work was supported by RF President Fellowship SP-259.2019.4. 1.Desfougeres, Y., et al., Charge and size drive spontaneous self-assembly of oppositely charged globular proteins into microspheres. J. Phys. Chem. B, 2010. 114(12): 4138-4144.

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Measuring composition and contact angles of reconstituted biomolecular condensates via quantitative phase microscopy
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Many compartments in eukaryotic cells are protein-rich biomolecular condensates formed via phase separation from the cyto- or nucleoplasm. A flurry of work over the last decade has identified many proteins sufficient to drive condensate formation as well as the conditions under which multi-phase equilibria are favored \textit{in vitro}, and semi-complete parts lists for a growing number of \textit{in vivo} condensates are becoming available. With this knowledge in hand, quantitative measurements of fundamental physical parameters like concentration and surface tension in reconstitution experiments can allow us to evaluate the thermodynamic and biochemical contributions of different condensate components. Although knowledge of condensate composition is essential for a full description of condensate properties and potential functions, existing quantitative measurements are scarce, due in part to a number of technical challenges. To address these, we use quantitative phase microscopy and optical diffraction tomography to measure the shape and refractive index of model reconstituted condensates resting on passivated glass substrates. From the refractive index and shape, we infer the absolute protein concentration and relative interfacial tension in individual condensates, respectively. Here, model condensates are formed by phase separation of purified protein constructs derived from members of the FUS/EWSR1/TAF15 protein family, with a focus on the primarily disordered RNA-binding domain (RBD) of TAF15. Surprisingly, we find that phase separation of TAF15(RBD) is attenuated only weakly by salt (0.05–3 M KCl) or temperature (10–50 °C), indicating that Coulombic and entropic interactions, respectively, play only minor roles in controlling the measured phase equilibria. Interestingly, we also find that partition coefficients determined by fluorescence microscopy dramatically underestimate protein concentrations in condensates, which we typically find to be 300–500 mg/ml. A simple model including inner filter and excited-state saturation effects suggests that the discrepancy stems primarily from reduced fluorescence quantum yields in condensates. Finally, we report progress in measuring the composition of reconstituted multi-component condensates containing more than one (bio)macromolecule by leveraging the high precision of refractive index measurements by quantitative phase microscopy to resolve the minute differences in optical dispersion of different polymers. Taken together, we provide needed experimental parameterization for polymer physics theories of protein phase separation, which will help elucidate the connection between the amino acid sequence and phase equilibria of reconstituted biomolecular condensates of increasing complexity.

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Crowding within the nucleus: TORC1 takes charge

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The properties of the cell interior are crucial for the organization and efficiency of biochemical reactions, but the physical nature of the cytoplasm, nucleus and other organelles remains poorly understood, particularly at the mesoscale (10 nm - 1 μm). One of the key and underappreciated property of the cell
interior is the high degree of macromolecular crowding. The diffusive and interactive behavior of molecules are significantly altered in a highly crowded environment, which in turn has a major impact on different biological processes and reactions. Macromolecular crowding is particularly relevant in the nucleus where large fraction of the volume is occupied by chromatin and the nucleolus. To date it remains a mystery how crowding within the nucleus is mechanistically regulated and what are the physiological consequences of perturbations to nuclear crowding. We have recently developed the nuclear-targeted Genetically Encoded Multimeric (GEM) nanoparticles that greatly facilitate the biophysical characterization of the cell. Key biophysical parameters including the degree of macromolecular crowding can be inferred from the motion of these probes. Using nuclear GEMs we show that inhibition of the TORC1 complex by rapamycin treatment decreases crowding within the budding yeast (*Saccharomyces cerevisiae*) nucleus. Genetic analyses revealed that downregulating rDNA transcription and ribosome biogenesis, a process that is tightly controlled by TORC1, phenocopies the effect of rapamycin treatment. Furthermore, TORC1 inhibition also decreased the size of the nucleolus, thus increasing the effective volume of the nucleoplasm. Therefore, we propose that TORC1 signaling adjusts molecular crowding within the nucleus by regulating rDNA transcription and/or the size of the nucleolus.

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**Natural selection on the level of molecular crowding in bacterial cells**  
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The dry mass dissolved in bacterial cells, comprised of proteins, metabolites, and other molecules, occupies a substantial fraction of the cytosolic volume. Increased substrate concentrations in crowded cells may boost cellular efficiency by raising biochemical fluxes. Crowding, however, also affects fluxes in other ways: (i) it may slow down the diffusion of a catalyst and its substrates, thereby reducing their encounter rates; (ii) it limits the available volume and thus reduces a solute’s entropy (*i.e.* the “excluded volume phenomenon”), thereby changing the Gibbs free energies of the molecules involved in the reaction and shifting the equilibrium concentrations of substrates and products. Hence, there exists an optimal density that maximizes biochemical efficiency and cellular growth; this optimal density depends on the size and shape distributions of the molecules. In nutritionally poor environments, the cytosol of *E. coli* is largely filled with metabolic enzymes and metabolites; in nutrient-rich environments, *E. coli* switches its pathways and the cytosol is instead dominated by the much larger ribosomes and tRNAs. This change in the size distribution of the cytosolic molecules may be responsible for a shift in cytosol density. Here, to understand how molecular crowding affects cellular efficiency at the network level, we simulated a simple model cell. This model has a metabolic pathway with small globular proteins that converts nutrients and small metabolites into precursor-metabolites, and a ribosomal pathway with large ribosome that binds with large tRNAs to synthesizes all biomolecular building blocks of the cell from the precursors. The growth rate of the model cell depends on the concentrations of the individual molecule species and the total cytosol density. This model accounts systematically for the slowdown of diffusion and the perturbation of Gibbs free energies through crowding. We find that the biochemical efficiency of reactions with larger catalysts and substrates is more sensitive to variations of the cytosol density. The theoretical dependence of the optimal cytosol density on the size distribution of the macromolecules is consistent with experimental observations of a ~10% difference in *E. coli* cytosol density between nutrient-rich and nutrient-poor environments. We conclude that the observed
dependence of cytosol density variation in E. coli is consistent with an optimality principle of cellular efficiency.

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The role of a transiently structured domain in controlling phase behavior of polyQ protein-RNA condensates

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Biomolecular condensates are a diverse class of membraneless, intracellular bodies that are likely formed via phase separation. Condensates often comprise proteins that are RNA-binding and predicted to have large disordered domains. A simple model of weak, multivalent interactions among disordered domains is commonly proposed as the driving force for protein phase separation, but avenues for regulation of these interactions are relatively uncharacterized. We have identified a metastable coiled-coil (CC) motif within the disordered polyQ tract of a phase separating protein that strongly influences the dense phase stoichiometries and material states of resulting RNA-protein condensates. The wild-type CC domain structure and mutants known to affect stability are predicted by atomistic monte carlo simulations and confirmed by circular dichroism. By incorporating our CC mutants into full-length proteins and combining with mRNA, we demonstrate the counterintuitive result that strengthening the CC interactions weakens the bulk protein-protein interactions in the system, altering dense phase composition and properties. Using coarse-grained particle models, we show that strong CC interactions sequester proteins into small oligomers distinct from the dense phase, and we provide experimental evidence in support of our model. Complementing previous work, we find that CC domains are about five times as likely to appear in polyQ-containing proteins than expected across the proteome, suggesting that the effects we have identified on polyQ protein phase behavior are common and generalizable. Finally, we use the Cahn-Hilliard equation coupled to a gelation process to propose that the apparent link between dense phase stoichiometry and material state is due to the intersection of ternary phase envelopes with log-parabolic sol-gel lines. This link between thermodynamics and condensate composition and viscoelastic properties represents a sensitive dial that cells could exploit during stress conditions or on evolutionary timescales.

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HP1α is a chromatin crosslinker that dictates nuclear and mitotic chromosome mechanics, morphology, and function

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Chromatin, which consists of DNA and associated proteins, provides not only the genetic information of the cell but also acts as a major mechanical component of the nucleus. Previous studies have shown that increasing histone methylation, a mark often associated with compact heterochromatin, increases
nuclear and mitotic chromosome stiffness. Constitutive heterochromatin also includes HP1α (CBX5), a protein unexplored as a mechanical component but that is implicated in heterochromatin function through compaction and/or as a crosslinker. We used a novel HP1α Auxin-inducible Degron (AID) cell line generated by the 4D Nucleome consortium to degrade HP1α in 4 hours and determine its role in nuclear and mitotic chromosome mechanics. Single nucleus isolation and micromanipulation force measurements reveal that HP1α is an essential mechanical component to chromatin-based short extension nuclear force response, while not contributing to lamin A-based strain stiffening at longer extensions. As with other perturbations that decrease chromatin-based nuclear rigidity, degradation of HP1α results in abnormal nuclear morphology, which is a hallmark of human disease. Nuclear mechanics and morphology are partially rescued in HP1α-depleted cells by increasing methylation of histones, suggesting that HP1α and histone methylation levels contribute independently. Rescue experiments reveal that HP1α mechanistically functions as a dimer/crosslinker, as the dimerization mutant HP1αI165E failed to rescue nuclear shape, contrary to HP1αWT. Simulations of nuclear mechanics recapitulate HP1α depletion as loss of chromatin-chromatin crosslinks, but not lamin-chromatin crosslinks. Furthermore, mechanical extension experiments on single mitotic chromosomes reveal that HP1α has a similar mechanical contribution to mitotic chromosomes that aids mitotic alignment and faithful segregation. Thus, HP1α is the first chromatin crosslinker protein shown to provide mechanical support to chromatin and aids key cellular functions.

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Molecular Transport through Reticulated Networks
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The endoplasmic reticulum (ER) forms an extensive network of interconnected sheets and tubules that plays a critical role in fundamental cellular functions such as protein manufacturing, secretion, and quality control, as well as calcium homeostasis. Many of these functions require the ER to serve as a delivery network for proteins, lipids, and ions throughout the cell. To date, the structure-function relationship between the complex morphology of the ER and its efficiency as a distribution center is poorly understood. Using computational models grounded in physical principles, we explore how the structure and dynamics of tubular ER modulate its ability to transport molecular components throughout the cell. Our results are parameterized and compared against live cell imaging data on the spreading of photoactivated proteins in the ER. We develop new quantitative methods for analysis of such data, and demonstrate that the spatiotemporal dynamics of photoactivated proteins are consistent with the presence of luminal flows that drive persistent motion of molecules between neighboring network nodes. We also show that narrowing of tubules associated with overexpression of the ER morphogen RTN4a results in substantially slower transport of proteins through the network. Using stochastic simulations, we probe the effect of putative luminal flows on the ability of the ER to replenish its calcium levels after a signalling event. Our results show that a combination of mobile buffer proteins and luminal flows of intermediate processivity allow for substantially faster restoration of calcium homeostasis in the ER lumen. Overall, our work demonstrates how the networked structure of the ER, and the presence of active flows within its lumen, allow it to function efficiently as a distribution and delivery network for intracellular components.
P604

**Understanding Liquid-Liquid Phase Separation in Crowded Cellular Environments**

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Recent studies have shown that liquid-liquid phase separation (LLPS) organizes cellular biochemical reactions through the formation of membrane-less organelles. Cells are extremely crowded, with up to 40% of the cell volume taken up by macromolecules. This molecular crowding can strongly affect LLPS. However, we still don’t fully understand the relationship between crowding and LLPS in cells. We investigated this problem by combining both experimental and computational studies of an engineered model LLPS system. We discovered that macromolecular crowding increases the kon rate of biochemical bond formation, shifts the critical point for LLPS, affects the kinetics of initial droplet formation, and stabilizes the condensed state. Our work highlights the potential importance of modulation of local or universal molecular crowding for LLPS.

P605

**Membraneless organelles at the origins of life?**

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Somewhere there was a habitat, hospitable for everything needed for the origins of life. The spaces between mica sheets may have been that habitat. Liquid-liquid phase separation between mica sheets may have brought together RNA and peptides to form ribosomes, as tiny membraneless organelles. Energy would be provided by mechanochemistry of moving mica sheets and the entropic energy of wet-dry cycles. Mica pieces in micaceous clay are large enough to support mechanochemistry from moving mica sheets. Living cells require metabolic cycles, replication of information, and protein synthesis. Life’s origins would have involved the following steps, in separate niches between mica sheets: 1) Evolution of metabolic cycles and nucleic acid replication; 2) Evolution of proteinsynthesis on ribosomes; 3) Repeated encapsulation by membranes of molecules formed in #1 and #2; followed by: 4) Interactions and fusion of membranes containing these molecules; resulting eventually in: 5) An occasional living cell, containing everything necessary for life. Mica sheets also provide hydrogen-bonding, enclosure, and more. The surfaces of adjacent mica sheets are bridged by exchangeable potassium counterions (K+), which are present at high concentrations in all living cells. The surfaces of mica (and clay) sheets have an 0.5-nm anionic crystal lattice, equal to the periodicity of phosphate groups in single-stranded nucleic acids. The surfaces of mica sheets could template nucleotide polymerization into linear oligonucleotides, as opposed to bent oligonucleotides with diphosphate linkages, for example. Black mica, biotite, is the best mica for life’s origins, because of its high Fe(II) content, capable of redox reactions needed for life’s origins. Maybe biology emerged from biotite. The origin of life is largely a historical science. We do experiments, but neither their success nor their failure proves how life did, or did not, emerge. Ideas are, therefore, valuable for investigating life’s origins, especially ideas that bring new research into the origins field, in new areas such as mechanochemistry and membraneless organelles. Hansma, H.G., Journal of Theoretical Biology, 2010. Origins of Life and Evolution of Biospheres, 2014. Life, 2017. Sci, 2020.
Post-Golgi Trafficking

P606

Elucidating the Role of Securin in Regulating Separase during Cortical Granule Exocytosis

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Meiosis is a tightly regulated series of events leading to the production of haploid gametes. A key player in this process is the cysteine protease separase. Known for its role in chromosome segregation, we have defined a role for separase in vesicular trafficking during cell division. Following spindle attachment and chromosome alignment during the meiotic M phase, the anaphase promoting complex/cyclosome (APC/C) is activated, resulting in the degradation of separase inhibitory chaperone securin and entry into anaphase I. At anaphase I onset, catalytically active separase proteolyzes a subunit of cohesin, allowing chromosomes to segregate. Separase also localizes to, and promotes exocytosis of, specialized vesicles called cortical granules through proteolysis of an unknown substrate. How separase localizes to sites with substrates is currently unknown. We hypothesize that cell cycle machinery known to control separase protease activity also controls its localization during anaphase I. During prometaphase, separase colocalizes with securin on kinetochores and mysterious filaments throughout the oocyte cortex that contain multiple kinetochore components. At anaphase I onset, separase transfers from the kinetochores to the space between the homologs where cohesin localizes. At the cortex, separase moves from filaments to vesicles. Securin is rapidly degraded during anaphase I and shows a faint localization to the midbivalent region. Inhibition of APC/C activity prevents separase and securin from leaving the filaments and kinetochores. Depletion of securin via RNAi causes premature localization of separase to cortical granules and blocks their exocytosis. These data suggest that the degradation of securin may allow separase to localize to sites with substrates during anaphase I. To test this further, we generated a non-degradable securin. Consistent with enhanced securin stability, non-degradable securin is not degraded following anaphase I onset and causes embryonic lethality. Non-degradable securin expression inhibits chromosome segregation, causes polar body extrusion failure, and blocks cortical granule exocytosis. Interestingly, non-degradable securin does not prevent separase localization to the midbivalent but delays separase localization to cortical granules until anaphase II. We conclude that the degradation of securin regulates separase localization to vesicles to coordinate exocytosis with chromosome segregation.

P607

Efficient export of single-spanning transmembrane proteins from the trans-Golgi network requires a fine-tuned transmembrane domain

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The Golgi complex is formed by a stack of biochemically-distinct flattened cisternae where secretory proteins are sequentially glycosylated before being sorted for their export from the trans-Golgi network (TGN). It has been suggested that this orderly sequence of events is facilitated by the lateral segregation of glycosylation enzymes from the export machinery. However, a complete mechanistic picture of this
The hydrophobic matching hypothesis suggests that single-spanning transmembrane proteins preferentially partition into membrane regions with a thickness that matches the hydrophobic length of the protein transmembrane domain (TMD). According to this, the short TMDs of Golgi-resident proteins promotes their segregation into thinner membrane regions for their recycle/retention, whereas secretory cargoes, which generally have longer TMDs, remain on thicker regions. However, this hypothesis has not been fully tested at the level of the Golgi complex. The high sphingolipid and cholesterol content of the trans-Golgi and TGN membranes contributes to the thickening of those bilayers, which are therefore poised for the hydrophobic matching mechanism to play a role in TMD-dependent protein sorting. To test this, we focused on two late-Golgi single-spanning transmembrane proteins: the trans-Golgi resident enzyme sialyltransferase (ST), and TGN46, a protein that cycles between the TGN and the plasma membrane. Since the TMDs of these proteins have a different length and amino acid composition, we were able to test how these TMD characteristics contribute to intra-Golgi localization. Our confocal and super-resolution microscopy (STED and STORM) data show that neither the length nor the amino acid composition of the TGN46 and ST TMDs dictate their intra-Golgi localization or glycosylation status. However, in the absence of specific luminal signals, the information encoded by the TMD becomes responsible for TGN46 intra-Golgi localization, indicating that the TMD may play a fine-tuning role on protein localization at the TGN. Several TGN export routes require the presence of sphingomyelin and cholesterol in the TGN membrane, suggesting that the export domains might be thicker and more laterally ordered. Interestingly, we found by FLIP microscopy that TGN46 mutants with shorter TMD domains exit the TGN with a slower rate than wild-type TGN46, suggesting that hydrophobic matching between the membrane and the cargo facilitates its sorting into export domains. Taken together, our data highlight the role of TMDs in assisting protein sorting and export at the TGN.

P608

Deciphering The Cellular Dynamics Of Acyl-Protein Thioesterase 2
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S-Palmitoylation is the only reversible post-translational lipid modification. It influences the localization, half-life and function of cellular proteins. Reversal of this modification is mediated by depalmitoylating enzymes, which include acyl protein thioesterase (APT) 1 and 2. While the structural characteristics of these two proteins have been well-studied, little is known about their intra-cellular dynamics. Here, we aim to decipher the in cellulo regulation of APT2. Fluorescence microscopy experiments indicate that APT2 accumulates at the Golgi apparatus, in addition to a diffused cytosolic staining and some presence at the plasma membrane. Previous experiments in our lab suggest that APT2 membrane association occurs via a three-step mechanism. Initially, APT2 is attracted to the membrane via electrostatic interactions followed by the insertion of a so-called β-tongue hydrophobic loop into the membrane. Once membrane bound, APT2 can be palmitoylated at Cys-2 by two protein acyl transferases, one which resides in the ER (zDHHC7) and the other on the Golgi (zDHHC3). This dual binding through the β-tongue and acyl chain is necessary for APT2 to exert its activity. The aim of this project is to understand how APT2 can access its targets on essentially all compartments of the endomembrane systems, including the plasma membrane, what are the dynamics of APT2 transport, and how it is controlled. To monitor spatio-temporal distribution of APT2 molecules, we have conducted fluorescence recovery after
photobleaching (FRAP) and photoconversion experiments. So far, the data indicate that APT2 can undergo palmitoylation on the ER followed by vesicular transport to the Golgi, or palmitoylation directly on the Golgi. While the APT2 transport from the ER appears relatively rapid, transport from the Golgi is slow and occurs by vesicular trafficking rather than depalmitoylation as previously proposed. Intriguingly, APT2 molecules show slow intra-Golgi dynamics suggesting a sub organellar compartmentalization that is under further investigation. Altogether, this study indicates that the distribution of APT2 through the endomembrane system is controlled by diffusion, palmitoylation, vesicular trafficking and depalmitoylation, and that this complex control of its localization is required for controlling depalmitoylation events within the cell.

P609

Discovery of a New Rab7A Secretory Pathway Involved in the Transport of Plasma Membrane Receptors.

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The localization of plasma membrane receptors is tightly regulated by different processes including secretion, endocytosis or recycling. The secretory pathway mediates the transport of proteins from the endoplasmic reticulum via the Golgi apparatus to the plasma membrane. To study the secretory pathway, the Retention Using Selective Hook (RUSH) system was recently developed (Boncompain et al., 2012). The RUSH system is a synchronized tool based on the use of a Hook linked to streptavidin to block a reporter of interest fused to a streptavidin binding peptide (SBP), at its site of biosynthesis. Release of this reporter using biotin allows the tracking of this protein to its specific trafficking pathway. Using this method, it was recently demonstrated that plasma membrane receptors could use distinct pathways to reach their final destination (Chen et al., 2017; Fourriere et al., 2019). However, the molecular partners involved in these processes are not clearly demonstrated. In our report, we engineered an edited version of the RUSH system (eRUSH) using CRISPR-Cas9, to decipher the molecular pathways involved in the trafficking of plasma membrane receptors. We used the Transferrin receptor 1 as a model of study in which we introduced at its C-terminal region a GFP and a SBP sequence (TfR-eRUSH). Combining mass spectrometry, siRNA screens and live imaging studies, we demonstrated the involvement of Rab7A in the transport of TfR-eRUSH. Using TIRF microscopy, we observed that Rab6A positive vesicles carry directly TfR-eRUSH to the plasma membrane, while Rab7A vesicles act like intermediate compartments for the transport of TfR-eRUSH. Characterization of these Rab7A-TfR-eRUSH vesicles demonstrated that they are distinct from acidic or degradative compartments which suggest a new role of Rab7A in the secretory pathway. Boncompain, G., Divoux, S., Gareil, N., de Forges, H., Lescure, A., Latreche, L., Mercanti, V., Jollivet, F., Raposo, G., and Perez, F. (2012). Nat. Methods 9, 493-498. Chen, Y., Gershlick, D.C., Park, S.Y., and Bonifacino, J.S. (2017). J. Cell Biol. 216, 4141-4151. Fourriere, L., Kasri, A., Gareil, N., Bardin, S., Bousquet, H., Pereira, D., Perez, F., Goud, B., Boncompain, G., and Miserey-Lenkei, S. (2019). J. Cell Biol. 218, 2215-2231.
P610

**Glycans Function as a Golgi Export Signal to Promote the Constitutive Exocytic Trafficking**

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Most proteins in the secretory pathway are glycosylated. However, the role of glycans in membrane trafficking remains unclear. Here, by abolishing or inhibiting O-glycosylation of transmembrane secretory cargos, such as interleukin 2 receptor α subunit or Tac, transferrin receptor and cluster of differentiation 8a, we found that they unexpectedly displayed obvious Golgi localization. After acquisition of their Golgi residence times, the observed Golgi localization of O-glycan deficient cargos was identified to be resulted from their slow Golgi export. Moreover, O-glycans were discovered to be both necessary and sufficient for the efficient Golgi export of our tested reporters and the effect of O-glycans on Golgi export is probably additive based on the quantitative study of O-glycans for ST6GAL1. Finally, artificially introducing one N-glycosylation site in the GFP tag of a Tac chimera substantially reduced its Golgi residence time, suggesting that N-glycans might exhibit a similar effect on Golgi export. Therefore, both O- and N-glycans might function as a generic Golgi export signal to promote the constitutive exocytic trafficking.

Protists and Parasites 2

P611

**A phosphoinositide-regulated telomeric expression site complex controls variant surface antigen expression in trypanosomes**

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The control of allelic exclusion and periodic switching of variant surface glycoprotein (VSG) genes is essential for African trypanosomes to evade the host antibody response during infection by antigenic variation. *Trypanosoma brucei*, a single-celled protozoan parasite, encodes 2,500 VSG genes; however, it only expresses one VSG gene at a time from one of the 20 telomeric expression sites (ESs). *T. brucei* changes the VSG coat by switching transcription between ESs or by VSG gene recombination. We identified a telomeric ES complex (TESC) of 0.9 megadalton that includes 24 proteins, and its VSG silencing function is controlled by a phosphoinositide regulatory system. In this complex, a nuclear phosphatidylinositol phosphate 5-phosphatase (PIP5Pase) enzyme associates with the repressor-activator protein 1 (RAP1). The knockdown of PIP5Pase or RAP1 results in transcription of all ES VSG genes, i.e. loss of VSG allelic exclusion. Using nanopore RNA-seq, we show here that parasites exclusively expressing a PIP5Pase catalytic mutant (D360A/N362A), which fails to dephosphorylate PI(3,4,5)P3, also express all ES VSG genes. Moreover, PIP5Pase was essential for VSG silencing in the insect stage forms, which do not express VSG genes. Chromatin immuno precipitate (ChiP) show that PIP5Pase and RAP1 interact with ES sequences flanking the VSG genes, namely 70 bp and telomeric repeats. We generated recombinant RAP1 as well as RAP1 domains BRCT (N-terminal), Myb (central), and Myb-like (MybL, C-terminal). We show that RAP1 binds to PI(3,4,5)P3 via its N-terminal BRCT domain. Moreover, rMyb and rMybL bind to synthetic 70 bp and telomeric repeats. Binding kinetics show that rRAP1 associates with telomeric and 70 bp repeats with dissociation constants (Kd) of 140 nM and 3.5 µM, respectively.
Notably, PI(3,4,5)P3 inhibits rRAP1 association with telomeric repeats and shift its Kd to 8 µM. To investigate how PI(3,4,5)P3 regulates RAP1 association with ES sequences in vivo, we performed ChIP analysis in parasites that exclusively express catalytic mutant PIP5Pase. The mutant cells show significant loss of RAP1 association with telomeric repeats compared to wild type cells. We propose a model in which PIP5Pase activity regulates RAP1 association with telomeric repeats, and thus silencing of VSG genes. Loss of PIP5Pase activity might result in the accumulation of PI(3,4,5)P3, which binds to RAP1 via the BRCT domain. The binding displaces RAP1 from the telomeric repeats, likely affecting ES chromatin organization, resulting in VSG transcription. The regulation of PIP5Pase activity might be essential for the control of VSG allelic exclusion and VSG developmental silencing.

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Beyond Cell Lines: Cytoplasmic Membrane Extensions in a Non-model Protist

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Cell-substrate interactions, motility, and communication through cytoplasmic membrane extensions are of vital interest in many contexts, as diverse as predator-prey interactions in microbial food webs, immune system function in animals, normal embryonic development, and metastatic migration of cancer cells. Studying the regulation of cytoplasmic membrane extension formation will further our understanding of this complex and medically important cellular process. Conventional and divergent cell biology features of protists make them a fertile and vastly understudied group for understanding eukaryotic biology. Here, we introduce an emerging model system, Aurantiochytrium limacinum that possesses unique cell biology features that are likely to offer insight into the origin and evolution of eukaryotic cytoplasmic membrane extensions. Particularly, A. limacinum uses an ectoplasmic network (EN) - branched extensions of the plasma membrane - to attach to substrates, gather food, travel, and communicate. The EN is associated with a unique cellular organelle called the bothrosome, and both are formed de novo when motile zoospores settle onto a surface and transition to growth as a vegetative thallus. Earlier microscopic characterization revealed the presence of an extensive network of actin filaments in the EN. However, information on the genes/proteins involved in the EN formation and its associated organelle is elusive. In our preliminary analysis, we have isolated soluble and insoluble protein fractions and found a number of consistent differences between EN-producing cells and non-EN forming cells (zoospores, and vegetative cells in suspension). For example, a shift in band density and size in the insoluble fraction between the zoospores and EN cells between ~50kDa (tubulin) and ~42kDa (actin) may represent the expected shift from tubulin in the flagella (which is resorbed) to actin in the EN. Using time-resolved confocal microscopy and live-cell imaging of actin polymerization, we visualized the EN formation. Settling A. limacinum zoospores change their morphology from a small flagellated cell to a large round cell which then produces EN within 5 hours. We are evaluating a Lifeact-mCherry fusion as a live reporter for actin dynamics in this process. Collectively, the dynamics of EN formation in A. limacinum will aid in understanding this biomedically important cellular process.
Three-dimensional organization and dynamics of the microsporidian harpoon-like invasion machinery


Microsporidia are obligate intracellular, spore-forming parasites that utilize a unique and specialized harpoon-like organelle called the polar tube (PT) to initiate infection. The PT is packaged within a dormant spore. Under suitable conditions, the PT shoots out of the spore as a linear tube, which the length of the tube can be ~20 times of its spore size. The PT is thought to penetrate the host cell membrane and serve as a conduit to transfer infectious cargo into the host. Microsporidian PT firing is one of the fastest known biological processes, and has not been well characterized. In this study, we address how the long PT is packaged inside a dormant spore, the kinetics of PT firing in different microsporidian species, and how the infectious cargo is transported through the PT. We used serial block-face scanning electron microscopy to reveal the three-dimensional architecture of the PT and its relative spatial organization to other organelles. Using high speed optical microscopy, we capture and quantify the kinetics of PT shooting in different species, as well as cargo movement through the PT. Our study sheds new light on the dynamics of the PT firing and unravels how cargo moves through the narrow PT.

MOB1 of *Toxoplasma gondii* regulates cell division rate and tachyzoite-bradyzoite conversion during the parasite’s asexual cycle

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*Toxoplasma gondii* is an obligate intracellular parasite of great veterinary and medical importance which transitions between several cellular stages during its life cycle. Notably, its ability to interconvert between fast replicating tachyzoite and slow replicating bradyzoite stages during its asexual cycle is instrumental for its success as a parasite. It permits parasite survival by establishing a chronic infection through tissue cysts that enable recrudescence. This conversion is dependent on cell cycle regulation and involves, among other events, cell differentiation and regulation of the division rate. MOB1 is a conserved kinase that regulates cytokinesis and division axis orientation in unicellular eukaryotes and also cellular proliferation versus apoptosis, centrosome duplication and cellular differentiation in multicellular eukaryotes. This led us to select MOB1 as a strong candidate to be involved in the *Toxoplasma* stage conversion process. We identified a single MOB1 gene in *T. gondii* and its mRNA levels drop abruptly after tachyzoite invasion and re-surge shortly before egress, suggesting a need for regulation of MOB1 mRNA levels during tachyzoite replication. We employed reverse genetics and CRISPR/Cas9 technology to assess the MOB1 gene function in *T. gondii*. Tachyzoites overexpressing MOB1 show a delay in the replication rate, supporting a role of MOB1 in the regulation of parasite
replication. In opposition to what has been observed in other unicellular eukaryotes, as *Tetrahymena thermophila* and *Trypanosoma brucei*, MOB1 knockout in *T. gondii* showed no cytokinesis impairment in its asexual cycle. Instead, we observed an increase in the replication rate of *T. gondii* and a significant loss in its ability to convert from tachyzoites to bradyzoites. The cytoplasmic localization of MOB1 recombinant protein and its accumulation at the end of mitosis, drawing a line between the two newly formed nuclei, suggests that this might be the cell cycle moment in which MOB1 is involved. To better understand MOB1’s role in the regulation of the *T. gondii* replication, we used a promiscuous biotin ligase BirA to identify the MOB1 interactome through nanoLC-MS/MS. Preliminary results identified conserved proteins related to DNA binding and to proteasome activity as well as coccidian specific proteins. Our data indicates that *T. gondii* MOB1 regulates both replication rate and tachyzoite-bradyzoite differentiation inside the parasitophorous vacuole. These features are closer to the MOB1 functions observed in multicellular eukaryotes, prompting us to regard the vacuole as an organ-like structure. Funding: this work was supported by Fundação para a Ciência e a Tecnologia (FCT), Portugal UID/CVT/00276/2019 and SFRH/BD/101619/2014 to ID.

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**Structural Basis of the Harpoon-Like Invasion Organelle of Microsporidia**

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Microsporidia are obligate intracellular, eukaryotic parasites capable of infecting a wide range of hosts from silkworms to immunocompromised humans. They do so through a fascinating organelle known as the polar tube which undergoes a rapid and tremendous conformational change from being tightly coiled inside the dormant spore to a long, linear tube out of the spore during infection. The filamentous polar tube is likely composed of repeating units of polar tube proteins (PTPs) whose structures remain a mystery, and which also don’t share sequence homology to any known protein. Furthermore, it is not well understood how these proteins are arranged to form the tube. An understanding of the protein structures, as well as how they are arranged to potentially form repeating units and information on the ultrastructure of the tube will be key to understanding the invasion mechanism. We have optimized sample preparation for cryo-tomography and observed two different populations of the polar tube of microsporidia species *Encephalitozoon hellem*, with different visual characteristics. We are currently applying subtomogram averaging to improve the resolution of our model and optimizing recombinant polar tube proteins for X-ray crystallography. By taking advantage of the advances made in cryo-electron tomography and combining it with X-ray crystallography we hope to understand how the architecture of the polar tube gives it robust material properties and facilitates infection.
Characterization of a eukaryotic initiation factor-2 alpha kinase in the reptilian protozoan parasite, *Entamoeba invadens*

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*Entamoeba invadens* is a reptilian intestinal parasite that is closely related to the human pathogen, *Entamoeba histolytica*. While these parasites possess similar life cycles and morphology, stage conversion of *E. histolytica* cannot be studied *in vitro*; therefore, *E. invadens* is used a model organism. In the host, the *Entamoebae* experience stress brought on by nutrient deprivation and the host immune response. To be successful parasites, these organisms must counter the stress; therefore, understanding the stress response may uncover new drug targets. In many systems, the stress response includes down-regulation of protein translation, which is regulated by the phosphorylation of eukaryotic initiation factor-2 alpha (eIF-2α). In mammalian cells, this phosphorylation is carried out by a family of four kinases, PERK (PKR-like ER kinase), PKR (protein kinase double-stranded RNA-dependent), GCN2 (general control non-derepressible-2), and HRI (heme-regulated inhibitor). While previous studies demonstrate the existence of this translational control pathway in the *Entamoebae*, no eIF-2α kinases have been characterized. In this study, we identified a putative *E. invadens* eIF-2α kinase, EiIF2K-A by searching the genome for eIF-2α kinase-like domains. Expression of EiIF2K-A in a heterologous yeast system demonstrated the ability of this protein to regulate growth via phosphorylation of eIF-2α. To further characterize protein function, we used trigger-mediated silencing to knockdown expression of EiIF2K-A and measured phosphorylation of eIF-2α and virulence functions. Reduced expression of EiIF2K-A led to significantly decreased levels of phosphorylated eIF-2α in trophozoites and encysting cells, suggesting that EiIF2K-A is an authentic kinase. Knockdown cells exhibited increased rates of encystation and decreased rates of excystation. Interestingly, virulence traits, such as phagocytosis and adhesion were significantly increased in knockdown cells compared to controls. Taken together, these data suggest that EiIF2K-A is an authentic eIF-2α kinase and plays a role in virulence.

RNA Localization and Transport

P617

**Determining long noncoding RNA mechanisms in stem cells using automated image analysis and single molecule RNA-Fluorescent In S itu Hybridization**

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Long noncoding RNAs (IncRNAs) are increasingly recognized as involved in human physiology and disease. Antisense IncRNAs are ubiquitous in the human genome and have many mechanisms of the action proposed, but the single-cell and single-molecule information that is needed to support or refute these mechanisms are lacking. Although single molecule RNA-Fluorescent In S itu Hybridization (RNA-FISH) is a single molecule method sometimes used to investigate IncRNAs, single-molecule counts and the spatial localization of these RNAs are often ignored. One reason is a lack of easily implemented and broadly applicable experimental and computational approaches for utilizing this data. We propose an RNA-FISH-based quantitative approach to test different mechanisms of gene regulation by antisense transcription. We applied our approach to investigate the mammalian antisense IncRNA pair Tsix and...
Xist known to have inhibitory interactions. By utilizing two-color, single-molecule RNA-FISH and quantitative image acquisition and analysis pipelines, we were able to quantify Tsix and Xist at single-molecule resolution in more than 8,000 cells including several biological replicas. Our analysis revealed that there was no significant colocalization of Xist and Tsix molecules, providing evidence against mechanisms of inhibition involving the binding of the two transcripts. We also found that anticorrelation between Xist and Tsix only occurred at sites of transcription, which suggests mechanisms of inhibition in cis based on the act of transcription. Surprisingly, our analysis of RNA-FISH data utilizing intronic probes provided evidence against transcriptional interference playing a significant role. Colocalization of transcription sites with histone marks elucidated possible relationships between active transcription and chromatin state. This data demonstrates the importance of single-molecule analysis when investigating noncoding RNA interactions. Our generalizable pipeline can be applied to transcripts across diverse species, cell types, and imaging modalities.

P618

**mRNA distribution in skeletal muscle is associated with mRNA size and microtubule-dependent**


Skeletal muscle myofibers are large and elongated cells with multiple nuclei evenly distributed. In multiple muscle disorders, nuclei are misplaced, but the relevance of nuclear positioning in disease is not clear. The distribution of nuclei suggests that each nucleus influences a specific compartment within the myofiber and implies a functional role for nuclear positioning. Compartmentalization of specific mRNAs and proteins has been reported at the neuromuscular and myotendinous junctions, but mRNA distribution in non-specialized regions of the myofibers remains largely unexplored. Using highly matured mouse myofibers differentiated *in vitro* and *ex vivo* myofibers, we observed that the bulk of mRNAs is enriched around the nucleus of origin. The perinuclear accumulation depends on newly transcribed mRNAs. By analyzing the distribution of several mRNAs using smFISH, we identified a subset of mRNAs that spreads throughout the myofiber. Both distribution patterns rely on microtubule transport away from the nucleus. We found that these distribution patterns are independent of nuclear dispersion, mRNA expression and stability, and the characteristics of the encoded protein. Surprisingly, the identified types of mRNA distribution correlate with mRNA size: normal size mRNAs are enriched in the perinuclear region whereas mRNAs encoding large proteins - giant mRNAs - are spread throughout the cell. Thus, we propose that mRNA distribution in non-specialized regions of skeletal muscle is transcript selective to ensure cellular compartmentalization and simultaneous long-range distribution of giant mRNAs.

P619

**Neural crest EMT is controlled via target degradation within P-bodies**

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Neural crest cells undergo a tightly regulated epithelial-to-mesenchymal transition (EMT) to delaminate from the neural tube. In the cranial neural crest, we have shown that this developmental EMT program is controlled by temporally restricted expression of the Wnt antagonist, Draxin. A hallmark of Draxin's
function during EMT is its transient expression and rapid downregulation. However, precisely how Draxin expression is regulated has been unclear. Using an in vivo reporter construct and time-lapse imaging to visualize RNA dynamics, we show that the rapid degradation of Draxin mRNA is mediated post-transcriptionally via its 3’-untranslated region (3’-UTR). We observed that Draxin transcripts are targeted to cytoplasmic processing bodies (P-bodies), which are membrane-less sites of RNA processing and decay. Through time-lapse imaging, we found Draxin mRNA not only co-localizes with a fluorescently-tagged P-body component (DCP1a), but is also rapidly dissolved within P-bodies in migrating neural crest. Furthermore, knockdown of the RNA helicase DDX6 via CRISPR/Cas9, known to dissolve P-bodies, disrupted compartmentalization of Draxin mRNA to P-bodies and it instead appeared broadly cytoplasmic. Importantly, disruption of P-bodies via DDX6 knockdown inhibited endogenous Draxin mRNA degradation and impeded cranial NC EMT in vivo. This work provides the first description of P-bodies in vertebrate neural crest through an adapted RNA live imaging approach and identifies an mRNA that is targeted to and degraded within P-bodies. Together, our data highlight a novel and important role for P-bodies in an intact organismal system—playing an essential role in neural crest EMT via targeted RNA decay. This work was funded by the US National Institutes of Health K99 DE028592 (EJH), K99DE029240 (MLP), R01DE027538, and R01DE027568 (MEB).

P620

Visualizing translation silencing by dynamic Ago2 tethering

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Translation regulatory factors are difficult to study in bulk. Ribosome profiling and more traditional bulk assays lack spatial and temporal resolution. In vitro assays lack the complex cellular microenvironment. Thus, some well-studied translation regulatory mechanisms remain ambiguous. One such mechanism involves the translation regulator Argonaute, which is essential for microRNA (miRNA)-mediated mRNA silencing. To overcome the limitations, we developed technology to directly visualize how Argonaute impacts the live-cell translation dynamics of single mRNA. Our methodology uses three complementary fluorescent probes to simultaneously track single mRNA, their translational status, and Argonaute tethering in real time. At the single-cell level, our system in steady-state confirms Argonaute-tetherable mRNA are fewer in number, less likely to be translated, and produce less mature protein than non-tetherable mRNA. At the single mRNA level, our system reveals heterogeneity in the type and spatial distribution of mRNA. Early on, a small, dynamic fraction (46%) of tethered mRNA were still translationally active. By tracking thousands of single mRNA, we were able to capture rare Argonaute-tethering events, which led to translational silencing in just 15 minutes on average. This timescale is similar to how long it takes a ribosome to translate the reporter ORF. This suggests Argonaute binding mainly blocks translation initiation rather than elongation. Though Argonaute-tetherable cells had less total reporter mRNA, whether Argonaute induced decay remains unclear. Translationally silent, Argonaute-tethered mRNA had a high propensity to accumulate in P bodies and persist for hours, in stark contrast to non-tetherable mRNA and control mRNA tethered to an inert protein, both of which did not accumulate in P bodies. Beyond Argonaute, researchers can now use our tethering technology to visualize in real time the impact of other proteins of interest on translating mRNA in live cells.
P621

**Investigating basal body-associated RNAs and their functions.**

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Despite the explosion of new methods to understand RNA on a genome-wide level, how RNA impacts large, cellular-scale machineries remains mysterious. Basal bodies (BBs) are one example of this untapped realm. These ancient macromolecular complexes nucleate cilia, and the protein composition and regulation of these structures has been the mainstay of BB research. However, little is known about how RNAs influence BBs. Early studies showed that RNA is indeed present and may promote BB structure and function, and more recent work shows that RNA is functionally important at the related structure, centrosomes. BB RNAs could influence the tightly regulated BB assembly pathway, the highly organized, cylindrical BB structure, or the crucial microtubule organization BB function. However, the identity of BB RNAs and therefore their roles in BB biology remains unknown. Here, we take a multi-pronged approach to address how RNAs influence BBs. First, we identified RNA binding proteins that interact with BBs, including Hsp90ab1, and tested its role in BB function and cortical organization. Second, we used an RNA sequencing approach to identify RNAs in close proximity to BBs. Finally, we used a candidate RNA approach to specifically identify whether mRNAs encoding BB proteins localize to BBs. Together, these studies support the hypothesis that RNAs localize to BBs and influence BB biology.

P622

**Morphologically-discrete, ER subdomains support the translation of different types of mRNAs in response to ER-lysosome interactions**

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The endoplasmic reticulum (ER) has a complex morphology comprised of stacked sheets, tubules, and three-way junctions, which together function as a platform for protein synthesis of the membrane and secretory proteins. It is believed that specific ER subdomains are spatially organized to provide protein synthesis activity, but precisely where these domains occur, especially with respect to a plethora of organelle interactions that take place on ER, remains elusive. Here, we use single-molecule tracking of ribosomes and mRNA in combination with simultaneous imaging of ER to assess the site of membrane protein synthesis on the ER. We found that ribosomes were widely distributed throughout the different ER morphologies, but the synthesis of membrane proteins (including Type I, II, and multi-spanning) and an ER luminal protein (Calreticulin) occurred primarily at three-way junctions. We found that Lunapark played a key role in stabilizing the transmembrane protein mRNA at the three-way junctions. Additionally, we found that these translating mRNAs are in the vicinity of the lysosome in an mTOR independent manner. These results support the idea that discrete ER subdomains co-exist with the lysosome to support specific types of protein synthesis activities with ER-lysosome interaction playing an important role in this organization.
Scaffolds and Complexes in Signaling

P623

**Pag1 directs src-family kinase intracellular localization to mediate receptor tyrosine kinase-induced differentiation**

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All receptor tyrosine kinases (RTKs) activate similar downstream signaling pathways through a common set of effectors, yet it is not fully understood how different receptors elicit distinct cellular responses to cause cell proliferation, differentiation, or other cell fates. We tested the hypothesis that regulation of SRC Family Kinase (SFK) signaling by the scaffold protein, Pag1, influences cell fate decisions following RTK activation. We generated a neuroblastoma cell line expressing a Pag1 fragment that lacks the membrane spanning domain (Pag1TM) and localized to the cytoplasm. Pag1TM cells exhibited higher amounts of active SFKs and increased growth rate. Pag1TM cells were unresponsive to TRKA and RET signaling, two RTKs that induce neuronal differentiation, but retained responses to EGFR and KIT. Under differentiation conditions, Pag1TM cells continued to proliferate and did not extend neurites or increase β-III tubulin expression. FYN and LYN were sequestered in multivesicular bodies (MVBs), and dramatically more FYN and LYN were in the lumen of MVBs in Pag1TM cells. In particular, activated FYN was sequestered in Pag1TM cells, suggesting that disruption of FYN localization led to the observed defects in differentiation. The results demonstrate that Pag1 directs SFK intracellular localization to control activity and to mediate signaling by RTKs that induce neuronal differentiation.

P624

**Multilayered mechanism regulating ERK1/2 signals transmitted through the Shoc2 scaffolding complex**

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Signaling scaffolds guide the flow of information and the spatial organization of the enzymes within the ERK1/2 signaling pathway. However, mechanisms that control assembly and dynamics within scaffolding complexes, as well as mechanisms regulating the cellular distribution of these complexes, remain largely unknown. We unravel a novel, multi-level paradigm in which allosteric modifications alter the ability of the scaffold protein Shoc2 to actively accelerate ERK1/2 signals. Shoc2 facilitates the ERK1/2 pathway by tethering to proximity essential enzymes of the pathway, Ras and RAF-1. Germ-line mutations in Shoc2 disrupting the spatial distribution of Shoc2 or its ability to assemble the signaling complex lead to a congenital disorder with a wide spectrum of developmental abnormalities, Noonan-like syndrome with loose anagen hair. Our lab studies mechanisms that fine-tune ERK1/2 signals transmitted through the Shoc2 complex. We found that Shoc2 assembles an elegant multi-component complex that incorporates several proteins of the ubiquitin system. To fine-tune amplitude of ERK1/2 signal transmitted via the complex, Shoc2 tethers the E3 ligase HUWE1, the (AAA+) ATPases, PSMC5 and VCP/p97 and the deubiquitinating enzyme, USP7. All of these enzymes are integral to the intricate feedback mechanism by which ubiquitination controls the amplitude of the Shoc2-ERK1/2 signals. Our studies show that while HUWE1 ubiquitinates Shoc2 and RAF-1, ATPases modulate the ubiquitination of Shoc2 and RAF-1.
through the remodeling of the complex. We demonstrated that PSMC5 and VCP/p97 are involved in the recruitment of the Shoc2 complex to endosomes where it undergoes remodeling. Importantly, our recent studies show that, in the context of the Shoc2 complex, USP7 functions as a “molecular switch”. In Shoc2 complex USP7 “activates” HUWE1 thereby triggering the mechanisms that “tunes-off” the ERK1/2 signals transmitted. Congenital Shoc2 mutations affecting Shoc2 interaction with USP7 lead to aberrant Shoc2 ubiquitination and signal transmission. In summary, our studies are the first to demonstrate that the Shoc2 scaffold employs multi-protein enzymatic machinery to govern the amplitude of Shoc2-ERK1/2 signals. We also uncover novel molecular mechanisms underlying the pathogenesis of Noonan-like syndrome with loose anagen hair. Overall, these studies significantly advance our understanding of the mechanisms by which non-enzymatic scaffolds regulate specificity and dynamics of the ERK1/2 signaling networks.

P625

The conserved GTPase Arf6 tethers Cdr2 nodes to the plasma membrane in fission yeast cells
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The plasma membrane serves as a platform to cluster signaling molecules into distinct signaling hubs.
In fission yeast, cell size control and cell cycle progression are connected through megadalton-sized signaling hubs termed “nodes” that are located on the plasma membrane. Nodes are established by the SAD family kinase Cdr2, which promotes mitotic entry by inhibiting Wee1. Cells lacking Cdr2 or other node proteins divide at a larger size than wild type cells due to overactive Wee1. Cdr2 contains a membrane-binding KA1 (kinase associated) domain, but other mechanisms likely contribute to anchoring of nodes to the membrane. Here, we performed a genome-wide screen for new components of the Cdr2-Wee1 signaling pathway. We found that the conserved GTPase Arf6 promotes mitotic entry through Cdr2-based signaling. Loss of Arf6 caused nodes to be disorganized on the membrane, resulting in defective phosphorylation of Wee1 and cell size at mitotic entry. These phenotypes were exacerbated when arf6Δ was combined with mutations that impair Cdr2 interactions with the membrane or with its binding partner Mid1. Remarkably, Cdr2 nodes detached from the plasma membrane in these mutants, resulting in node-like clusters within the cytosol. Consistent with the notion that Arf6 acts as a regulated anchor for nodes, we found that Arf6 co-localized to Cdr2 nodes in a manner that depended on its GTPase cycle. Because Arf6 is conserved throughout eukaryotes, our results suggest a new role for this GTPase as a regulated anchor for signaling clusters at the plasma membrane. In addition, we provide a new model for how Cdr2 nodes remain tethered at the plasma membrane to promote mitotic entry at a reproducible cell size.

P626

Proximity dependent labeling of Abelson interactor 1 (Abi-1) reveals its involvement in centrosome biology, inflammatory signaling, and membrane trafficking
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Background: Abelson interactor 1 (Abi-1) is a signal-facilitating adaptor protein regulating cytoskeletal remodeling, cell growth and adhesion. The role of Abi-1 in carcinogenesis is unclear as both elevated
Abi-1 and decreased expression were reported in solid tumors, highlighting an essential role of Abi-1 in cellular homeostasis. Our group reported that loss of Abi-1 in the hematopoietic system results in development of Src Family Kinases/NF-κB/STAT3-driven myeloproliferative neoplasm (MPN). Decreased levels of ABI1 transcripts were found in MPN stem/progenitor cells. To provide mechanistic insight to the Abi-1 interactome we employed proximity dependent biotin labeling followed by mass spectrometry (PDBL/MS) enabled by TurboID and label-free quantitation. **Method:** Single-cell derived, stable NIH/3T3 cell clones expressing TurboID-Abi-1-IRES-GFP (TurboAbi) or TurboID-IRES-GFP (TurboControl) were established. Wildtype NIH/3T3 (WTControl) was used as an additional control. Three separate biotin labeling experiments were performed. To determine most probable Abi-1 interactors, we used peptide spectral match (PSM) and signal intensity peak area (PA) to calculate enrichment ratios and FDRs for streptavidin-enriched proteins identified in TurboAbi vs. controls as well as average number of PSMs identified in TurboAbi group. An RShiny app was developed to assist analysis and interpretation of the results. **Results:** 4,082 proteins were identified, with 2,997 proteins identified in TurboAbi group. Using statistical threshold: TurboAbi vs. controls FDR ≤ 0.05, enrichment ratio ≥ 1.5 for PA and PSM and minimum average 1 TurboAbi PSM per injection, we identified 212 probable Abi-1 interacting proteins (32 known). Using these statistical considerations, we identified known interactors (e.g. WAVE2, NAP1, SRA1) as well as new Abi-1-interacting complexes including pericentriolar material scaffolding components CEP152, AZI1 and PCM1, cell-death regulating TRIKA2 complex comprising MAP3K7/TAB2/RIPK1 as well as proteins regulating intracellular trafficking including EPS15R, ITSN2 and SYNJ1. **Conclusions:** Using PDBL/MS technology we established the Abi-1 interactome and identified, in addition to known Abi-1 roles, new functions of Abi-1 in centrosome biology, inflammatory signaling and membrane trafficking. Obtained data will aid identification of new pharmacological targets in MPNs.

P627

The A-kinase anchoring protein synemin binds PKCe

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Stimulation of β-adrenergic receptors (β-AR) in cardiomyocytes results in activation of two parallel pathways: the cAMP/PKA pathway and the cAMP/Epac/PKCe pathway. Given the importance of β-AR/- cAMP pathways in various cardiomyopathies, it is critical to understand the role of each under normal conditions. Synemin is an intermediate filament protein; our early work revealed it also functions as a scaffold protein for PKA, classifying it as an A-kinase anchoring protein (AKAP). AKAPs act to tether PKA to specific subcellular locations to maintain the specificity of the PKA signaling pathway. The two largest isoforms (α and β) are usually co-expressed in various tissues such as cardiomyocytes. Excitingly, we have found evidence that synemin also binds PKCe. Yeast two-hybrid analysis showed both α- and β-synemin can interact with PKCe. Furthermore, β-galactosidase liquid assays revealed that the relative strength of interaction between α-synemin and PKCe is significantly stronger than that of β-synemin and PKCe. In vivo studies demonstrated that β-synemin can bind to PKCe in vivo. Specifically, HL-1 cells (a cardiac muscle cell line) were transfected with cDNA encoding epitope tagged, wildtype or mutant β-synemin; after 48 hours the cells were treated ± isoproterenol to stimulate the β-AR pathway. Co-immunoprecipitation studies revealed that PKCe binds to β-synemin but only upon stimulation of the β-AR pathway. In addition, similar studies showed that PKCe does not co-immunoprecipitate with mutant β-synemin carrying two missense mutations (mutant β-synemin is unable to bind PKA but is still able to form intermediate filaments). Taken together our data suggests that synemin is a site of crosstalk of the
PKA and PKCε pathways. More precisely, our findings suggest that PKCε translocates to synemin upon activation of the β-AR. Furthermore, the data indicate that PKCε either binds at/near the same location as PKA on β-synemin or that PKA binding/activation is a prerequisite for PKCε binding to β-synemin. Recently discovered mutations in synemin have been linked to dilated cardiomyopathy and a rare syndrome that affects the heart (ulnar-mammary-like syndrome). Therefore, characterization of pathways that intersect at synemin will likely reveal details of synemin-organized signaling in the heart. Such details could offer leads on potential therapeutic targets.

P628

**Signalsome nucleation enables digital innate immune proinflammatory responses**

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Pathogen sensing by innate immune cells results in binary all-or-none proinflammatory responses. These responses are coupled to the formation of polymeric protein self-assemblies known as signalosomes. However, whether signalosome formation is directly responsible for binary signaling remains unclear. We have previously found that the formation of certain signalosomes involves a rate-limiting nucleation step. Here, we evaluated the existence and functional consequences of a hypothetical nucleation barrier associated with assembly of the CARD9-Bcl10-MALT1 (CBM) signalosome, which activates transcription factor NF-κB upon fungal pathogen sensing. We employed Distributed Amphifluoric FRET (DAmFRET), a recently developed flow cytometry-based method that determines the frequency of protein nucleation in cells using flow cytometry. We determined that CARD9 and Bcl10 form nucleation-limited polymers with saturating concentrations similar to those from in vitro studies. We then dissected the nucleation steps required for signalsome formation. Co-expression of artificially oligomerized CARD9 robustly nucleated Bcl10 to its polymeric form. Remarkably, CARD9 oligomers containing mutations that impair antifungal signaling in humans were unable to nucleate Bcl10, suggesting that the nucleating activity of CARD9 is essential for signaling. To address whether the nucleation-limited polymerization of Bcl10 causes binary activation of NF-κB, we introduced a single cell fluorescent transcriptional reporter of NF-κB activity into the DAmFRET assay. This approach revealed that cells containing Bcl10 assemblies strongly induced NF-κB activation, while cells expressing the same level of Bcl10 in its pre-assembled monomeric state did not. We further showed that rational point mutations that increase or decrease the nucleation barrier for Bcl10 correspondingly impaired or enhanced, respectively, NF-κB activation. Our results indicate a critical role of nucleation barriers in governing the functional outcome of signalosomes. This line of inquiry could uncover a fundamental molecular mechanism of innate immune signaling, which will inform our understanding of the causes of autoinflammatory diseases.

P629

**Adaptor Binding Position in a Signalling Network Impacts Actin Polymerisation**

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Phosphotyrosine (pTyr) motifs recognised by SH2 domains are central to innumerable cellular signalling pathways including those regulating T cell activation and EGF receptor function. pTyr-SH2 interactions
are often dysregulated in cancers. Their ubiquity and modularity also make them highly relevant in synthetic signalling systems. pTyr motifs frequently occur in poorly ordered regions of proteins as multiple sites binding different SH2-containing adaptors. Current models posit that phase separation of disordered, multivalent signalling proteins is important for their function. However, the rules if any, for assembling such complex networks are not known. The impact of pTyr arrangement in signalling proteins on downstream function is also unknown. We examine the importance of motif positioning in an in vivo model for pTyr signalling. Vaccinia virus egressing from host cells activates Src and Abl family kinases to phosphorylate A36, an integral membrane viral protein that is largely unstructured. A36 pTyr 112 and 132 motifs bind the SH2 domains of adaptors Nck and Grb2 respectively. These adaptors interact with WIP:N-WASP which in turn activate the Arp2/3 complex. The resulting actin polymerisation can be visualised as a comet tail that drives virus motility and enhances cell-to-cell spread of Vaccinia. Nck is essential for actin tail formation, while Grb2 recruitment stabilises the signalling complex. We constructed recombinant viruses where A36 was edited to exchange the positions of these pTyr motifs. The resulting viruses demonstrate a striking impairment of actin polymerisation and associated virus motility. Interestingly, while the levels and stability of Nck remain unchanged, Grb2 is very poorly recruited to the modified virus. We could partially restore actin polymerisation by adding an extra Grb2-binding motif C-terminal to the Nck site but not N-terminally. These dramatic differences observed in signalling output imply that the relative positioning of adaptor binding motifs in disordered proteins is unlikely to be arbitrary. We are currently investigating the mechanism underlying our observations and expanding our findings to other pTyr signalling networks.

P630

Visualisation of IL1 inflammatory signaling reveals the stepwise assembly of MyD88, IRAK4 and IRAK1 into Myddosomes

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The adapter MyD88 connects IL1R/TLR receptors to downstream signalling cascades that activate an innate immune response. Biochemical models of IL1R/TLR signalling have limited consideration for the dynamics of MyD88 and how it associates with IRAK family kinases to signal via an oligomeric complex termed the Myddosome. Here we have developed a live cell approach to visualise IL1R and Myddosome signalling. We find that IL1 engagement with IL1R induces the nucleation of MyD88 oligomers at the plasma membrane. MyD88 oligomerization is initially reversible. However, MyD88 oligomer stability increased with size. The formation of larger MyD88 oligomers consisting of >4 monomers triggered the sequential recruitment of IRAK4 and IRAK1. IRAK4 knock out cells formed larger MyD88 oligomers and revealed that IRAK4 caps the growth and size of MyD88 oligomers. This data reveals Myddosome assembly is inducible, and that a critical size of MyD88 oligomers is required to trigger downstream signalling. These results show the fundamental role of triggered macromolecular assembly in IL1R signalling. Furthermore, these results reveal how macromolecular assembly and protein oligomerization functions in cellular signaling pathways.
P631

Characterizing the function of heparan sulfate proteoglycans and the novel heparin receptor TMEM184A in a mechanosensing complex
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TMEM184A is a novel heparin receptor in the vasculature that decreases cell proliferation and inflammatory signaling with heparin binding. When exposed to unidirectional, laminar fluid shear stress (fss), endothelial cells align in the direction of the flow. TMEM184A co-localizes with VE-Cadherin and VEGFR2, and its expression is upregulated when cells are exposed to fss. Additionally, endothelial nitrous oxide synthase (eNOS) is activated in response to heparin binding to TMEM184A, and this activation appears to occur at focal adhesion sites. Preliminary evidence suggests potential integrin involvement in this process. Heparin induced eNOS activation is dependent on TMEM184A. Together, this suggests a mechanosensing role for TMEM184A in adherens junctions. This study aims to elucidate the relationship between TMEM184A and mechanosensing proteins: Syndecans (Sdc) 1, 2 and 4, VE-Cadherin, and VEGFR2. Upon onset of fss, Sdc4 and TMEM184A begin to colocalize, and after 30 minutes of flow TMEM184A colocalizes with VEGFR2 and VE-Cadherin. Data in the literature found that endothelial glycocalyx deficient in HSPGs demonstrates a failure to align in the direction of flow and that heparanase treatment inhibits HSPG induction of VE-Cadherin, implicating HSPGs in mechanotransduction. TMEM184A may play a role in trafficking HSPGs, specifically Sdc1 and Glypican 1. There is evidence of membrane colocalization between Sdc1 and TMEM184A in immunofluorescence (IF), and Sdc1 appears to localize in puncta above the nuclear region, further supporting a trafficking role. Immunoprecipitation (IP) of TMEM184A precipitates Sdcs 1 and 2. We hypothesize a potential linking role for HSPGs, linking TMEM184A to the adherens junction mechanosensing complex. Using IF, western blot analysis, and co-IP we have shown TMEM-VE-Cadherin and TMEM-Sdc 1 and 2 protein-protein interactions in bovine aortic endothelial cells. shRNA mediated knockdown of TMEM184A in rat aortic endothelial cells leads to observed decreases in VE-Cadherin in adherens junctions, while TMEM184A overexpression increases VE-Cadherin membrane populations. High salt disrupts interactions with HSPGs and decreases colocalization between VE-Cadherin and TMEM184A in IF. The goal of this work is to elucidate how HSPGs are interacting with TMEM184A and characterize the function of this relationship in mechanosensing complexes. We have significant evidence for interactions between Sdcs and TMEM184A, and between TMEM184A and other mechanosensing proteins. It is clear that TMEM184A and HSPGs work together to modify endothelial layer function, however it is yet to be established whether interactions are dependent on HS chains, or if they work together in another capacity to modulate endothelial function.

P632

A hypothetical MEK1-MIP1-SMEK multiprotein signaling complex may function in Dictyostelium and mammalian cells.
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In our previous study we characterized Dictyostelium SUMO targeted Ubiquitin Ligase (StUbl) MIP1 that associates with protein kinase MEK1 and targets SUMOylated MEK1 to ubiquitination and proteasomal degradation. These modifications happen in response to activation of MEK1 by chemoattractant cAMP.
SMEK - second site genetic suppressor of mek1- null phenotype also identified in Dictyostelium. MEK1 and SMEK belong to the same linear pathway, in which MEK1 negatively regulates SMEK, which then negatively regulates chemotaxis and aggregation. RNF4 is mammalian homologue of MIP. RNF4 interacts with human homologue of Dictyostelium SMEK - hSMEK2. We propose existence of evolutionarily conserved MEK1-SMEK signaling complex that upon MEK1 activation and SUMOylation, recruits Ubiquitin Ligase MIP1/RNF4, which, in turn, ubiquitinates SMEK and targets this protein for proteasomal degradation. This could be a mechanism for negative regulation of SMEK by MEK1 signaling.

Tubulin Post-Translational Modifications

P633

How Enzymes add Short and Long Glutamate Chains to Tubulin Tails to Functionalize Microtubules

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Glutamylation, introduced by TTLL enzymes, is the most abundant modification of brain tubulin. Essential effector proteins read the tubulin glutamylation pattern, and its misregulation causes neurodegeneration. TTLL glutamylases posttranslationally add glutamates to internal glutamates in tubulin C-terminal tails (branch initiation, through an isopeptide bond), and additional glutamates can extend these (elongation). TTLLs are thought to specialize for initiation or elongation, but the mechanistic basis for regioselectivity is unknown. We present cocrystal structures of murine TTLL6 bound to tetrahedral intermediate analogs that delineate key active-site residues that make this an elongase. We show that TTLL4 is exclusively an initiase, and through combined structural and phylogenetic analyses, engineer TTLL6 into a branch-initiating enzyme. TTLL glycylases add glycines posttranslationally to internal glutamates, and we find that the same active-site residues discriminate between initiase and elongase glycylases. These active-site specializations of TTLL glutamylases and glycylases ultimately yield the chemical complexity of cellular microtubules.

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Evolutionary significance of tubulin arginylation

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The tubulin molecule is subject to a very large number of post-translational modifications, some of which are very rarely found in other proteins. Recently our data from proteomic screens suggested that arginyl transfer enzyme 1 (ATE1) can add an arginine to both α-tubulin (D69, E77, E420, and E429) and β-tubulin (E69, D74, D118 and D295). Furthermore, we found that ATE1 deletion carries functional consequences for microtubule properties in vivo. It is not yet clear what roles the individual arginylated residues play in this process, although they are all widely conserved in the evolution of the individual α- and β-tubulin families. We here examine the unusual conservation of the site at position 69
that is common to both α- and β-tubulin. In α, positions 68-72 (VDLEP) are the same as positions 66-70 in β (VDLEP), the difference being that in α, the arginylated residue is D and in β it is E. These regions are conserved virtually throughout eukaryotes, with the exception that the initial V is occasionally replaced by L or M, which, like V, are hydrophobic. The same VDLEP sequence is also seen in prokaryotic Prosthecobacter BtubA and BtubB, which are thought to have arisen from a lateral transfer from a common eukaryotic ancestor to α- and β-tubulin. If this sequence has an affinity for ATE1, which is itself a highly conserved enzyme, then it is possible that either the D or the E was arginylated prior to the appearance of microtubules. Looking at other tubulins, there are greater divergences in this region, although the relative topography of D and E remain the same: human γ (LDLEP), human δ (VDMEP), Volvox η (VDTEP), Trypanosoma ζ (VDSEP), Paramecium θ (IDLEP), Paramecium ι (VDSEE), and Paramecium κ (IDSES). In contrast, the prokaryotic relatives of tubulin (FtsZ, TubZ and RepX) have no related sequences. In short, it appears that this region has remained essentially unchanged since before microtubules appeared and perhaps even before α-, β-, γ- and δ-tubulin diverged and may have originated at the time of the first eukaryotes. If the reason for this conservation was to preserve arginylation sites, then one must ask what the role of such arginylation would have been. It would be worth examining whether these various forms of tubulin are arginylated and, if so, what the functions of this arginylation might be.

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A phospho-regulated signal motif determines subcellular localization of α-TAT1 for dynamic microtubule acetylation

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Background: Microtubule (MT) acetylation is a highly conserved post-translational modification that stabilizes MTs and mediates responses to mechanical forces at molecular, cellular and organism levels. MT acetylation underlies many physiological events ranging from cell migration to tissue stiffness, intracellular trafficking, autophagy and viral infections. MT acetylation is exclusively catalysed by α-TAT1, for which the only known substrate is α-tubulin. While the functional consequences of MT acetylation have been extensively studied, the molecular mechanisms that regulate α-tubulin N-acetyltransferase 1 (α-TAT1) action remain obscure. Results: We have utilized computational sequence analyses and mutational analyses to identify and characterize a conserved signal motif in the intrinsically disordered α-TAT1 C-terminus that mediates its intracellular distribution. This signal motif consists of three functional modules: a nuclear export sequence (NES), a nuclear localization sequence (NLS), and phosphorylation sites. Using live cell microscopy, we observed that α-TAT1 shuttles between the nucleus and cytosol but is mainly cytosolic due to CRM1-mediated nuclear export. Nuclear localized α-TAT1 catalytic domain did not induce MT acetylation, suggesting that cytosolic localization of α-TAT1 mediates its function. We show that the α-TAT1 NLS is phospho-inhibited by cyclin dependent kinases, Protein Kinase A and Casein kinase 2, and that the phosphorylated motif binds to 14-3-3 adaptor proteins to retain α-TAT1 in the cytosol for maximal substrate access. We hypothesized that inducible nuclear transport α-TAT1 will allow control of MT acetylation. We modified the previously described Light-inducible nuclear export system (LEXY) to reduce its dark-state activity and tethered it to the α-TAT1 catalytic domain. LEXY- α-TAT1 localized to the nucleus under dark conditions and was rapidly and
reversibly shuttled to the cytosol on blue light stimulation. HeLa cells expressing LEXY- α-TAT1 exposed to light showed increased MT acetylation unlike those kept under dark conditions, thus validating the functionality of the tool. **Conclusions:** Our findings establish a novel role for the intrinsically disordered C-terminus of α-TAT1 in its function by regulating its intracellular localization downstream of kinase and phosphatase activities. Moreover, we have developed a powerful tool to control MT acetylation that will provide new applications that can be used to elucidate the role of MT acetylation in health and diseases.

**P636**

**Investigations of Microtubule Stability in Spinal Muscular Atrophy Patient Fibroblasts**

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Spinal muscular atrophy (SMA) is an inherited neurodegenerative disease and the most common genetic cause of infant mortality. Survival of motor neuron 1 (SMN1) gene mutations lead to the absence of functional SMN protein, which results in lower motor neuron loss in the spinal cord and progressive muscle atrophy. SMN deficiency causes alterations in all cytoskeletal elements and regulatory signalling pathways, both have been associated with defective neuronal morphology as well as axonal transport. Our previous findings indicated significant alterations in some microtubule associated proteins and also alpha tubulin post-translational modifications, suggesting altered microtubule stability in an in vitro and in vivo SMA model systems. In this study, we utilized patient fibroblast cells to investigate microtubule stability in vitro. Acetylated alpha tubulin level was analyzed as stability marker in fibroblast cells of two clinically different SMA patients (type I and II) as well as healthy controls. Western blot and immunofluorescence studies showed a significant reduction in acetylated alpha tubulin level in severe type I patient cells. Additionally, we compared proliferation rates of patient and control cells by MTS assay and found an increase in proliferation of patient cells. Our findings suggest that in addition to in vitro and in vivo SMA models, microtubule dysregulations are also exist in patient fibroblast cells. Studies are ongoing to understand the effects of SMN reduction on microtubule stability and underlying mechanisms. This study is supported by Hacettepe University Scientific Research Projects Coordination Unit (Project number: TYL-2019-18351) **Keywords:** Spinal muscular atrophy, microtubule, post-translational modifications

**P637**

**Polyglutamylation of microtubules drives motor axon remodelling**

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Microtubules play a key role in cellular homeostasis, polarity and transport. Microtubules are dynamic tube-like structures, build from dimers of differentially expressed α- and β-tubulin subunits—a basic structure that could carry a range of post-translational modifications (PTMs). Together with associated proteins, PTMs establish a ‘code’, which endows the microtubular scaffold with specific and local functionality, such as regulating microtubule length and dynamics, but also microtubule-dependent transport. However, how this code translates into shaping cell- or even tissue-level events, e.g. during
development, remains largely unexplored. Here, we investigated how polyglutamylation, a PTM enriched on neuronal microtubules, regulates mammalian motor axon remodelling. During early postnatal development the vast majority (~90%) of terminal axon branches are pruned by a regressive process that involves spastin-mediated loss of microtubules. While spastin is known to preferentially sever polyglutamylated microtubules, it remains elusive whether local polyglutamylation is a driver of axon pruning or just an epiphenomenon. To address this, we used motor neuron-specific deletions of glutamylases and deglutamylases, which act as ‘writers’ and ‘erasers’ of glutamylation patterns on tubulin tails. Consistent with an instructive role of polyglutamylation, motor neurons ablated of CCP1&6 deglutamylases accelerated axon pruning, while deletion of TTLL1—which elongates seeded polyglutamate chains—delayed axonal remodelling. In ongoing experiments, we are measuring polyglutamylation, as well as microtubule mass and dynamics in situ. Our preliminary data largely corroborate the predicted local regulation of microtubular stability, which could be indicative of a rheostatic regulation of severing and hence pruning by TTLLs and CCPs. Surprisingly, we also discovered that deletion of TTLL7—which ‘seeds’ glutamate chains on alpha tubulin—had no effect on total polyglutamylation, suggesting further levels of regulation that are engrained in parallel or consecutive steps of editing the tubulin code. In summary, our data point to a model where a specific tubulin PTM—polyglutamylation—acts as an instructive signal for spastin-mediated severing, which in turn paces developmental axon pruning. Our data thus show that the ‘tubulin code’ can be used to control specific morphogenetic events during nervous system development. This will motivate future work to explore how polyglutamylation is locally regulated and whether similar mechanisms determine axon stability in central neurons and during disease-related remodelling.

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**Novel alpha-tubulin acetylation site controls microtubule dynamics to regulate neuromuscular junction development**

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Microtubules are essential for cells to function. This is especially true of neurons, where disrupting microtubule growth, stabilisation, or catastrophe perturbs neuronal morphology and function. Long-lived microtubules form the stable frame of a neuron whilst dynamic microtubules allow a neuron to grow, remodel and adapt to its surroundings. The post-translational modifications (PTMs) of an otherwise homogeneous population of tubulin have the potential to directly tune microtubule stability and dynamics. Large-scale proteomic studies have identified multiple acetylation sites in α-tubulin that have, as yet, unknown roles in regulating microtubule function. Our mass spectrometry analysis of tubulin purified from rat cortical neurons identified four such acetylation sites in α-tubulin. Using Drosophila as a model system, K-to-A mutagenesis of endogenous α-tubulin shows that two of the four conserved sites are essential. Based on its position at the αβ-tubulin dimer interface, we hypothesise that acetylation of one of these uncharacterised residues may regulate the stable addition of αβ-dimers onto microtubules and thus overall microtubule stability. Consistent with this idea, an acetylation-blocking mutation (K-to-R) results in fewer stable microtubules at the neuromuscular junction. We can rescue the loss of stable microtubules by over-expressing a microtubule-associated protein, Futsch, that stabilises microtubules. Over expression of a tubulin chaperone protein that is involved in forming a functional αβ-tubulin dimer and managing the free dimer pool is also able to rescue the stable
microtubules suggesting that when we block acetylation there is an increase in free dimer due to a decrease in dimer stability. Together, these results suggest that acetylation may be balancing microtubule dynamics and stability by regulating dimer stability on dynamic microtubules. Intriguingly, mutating this α-tubulin residue results in two distinct morphology phenotypes in the axon terminals of two different neuron types. This raises the enticing possibility that microtubule dynamics and stability are differentially tailored in different neuron types to create synapses that have distinct morphologies and functions.

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A phosho-regulated signal motif determines subcellular localization of α-TAT1 for dynamic microtubule acetylation
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Background: Microtubule (MT) acetylation is a highly conserved post-translational modification that stabilizes MTs and mediates responses to mechanical forces at molecular, cellular and organism levels. MT acetylation underlies many physiological events ranging from cell migration to tissue stiffness, intracellular trafficking, autophagy and viral infections. MT acetylation is exclusively catalysed by α-TAT1, for which the only known substrate is α-tubulin. While the functional consequences of MT acetylation have been extensively studied, the molecular mechanisms that regulate α-tubulin N-acetyltransferase 1 (α-TAT1) action remain obscure. Results: We have utilized computational sequence analyses and mutational analyses to identify and characterize a conserved signal motif in the intrinsically disordered α-TAT1 C-terminus that mediates its intracellular distribution. This signal motif consists of three functional modules: a nuclear export sequence (NES), a nuclear localization sequence (NLS), and phosphorylation sites. Using live cell microscopy, we observed that α-TAT1 shuttles between the nucleus and cytosol but is mainly cytosolic due to CRM1-mediated nuclear export. Nuclear localized α-TAT1 catalytic domain did not induce MT acetylation, suggesting that cytosolic localization of α-TAT1 mediates its function. We show that the α-TAT1 NLS is phospho-inhibited by cyclin dependent kinases, Protein Kinase A and Casein kinase 2, and that the phosphorylated motif binds to 14-3-3 adaptor proteins to retain α-TAT1 in the cytosol for maximal substrate access. We hypothesized that inducible nuclear transport α-TAT1 will allow control of MT acetylation. We modified the previously described Light-inducible nuclear export system (LEXY) to reduce its dark-state activity and tethered it to the α-TAT1 catalytic domain. LEXY-α-TAT1 localized to the nucleus under dark conditions and was rapidly and reversibly shuttled to the cytosol on blue light stimulation. HeLa cells expressing LEXY-α-TAT1 exposed to light showed increased MT acetylation unlike those kept under dark conditions, thus validating the functionality of the tool. Conclusions: Our findings establish a novel role for the intrinsically disordered C-terminus of α-TAT1 in its function by regulating its intracellular localization downstream of kinase and phosphatase activities. Moreover, we have developed a powerful tool to control MT acetylation that will provide new applications that can be used to elucidate the role of MT acetylation in health and diseases.
**Tumor Invasion and Metastasis: Cell signaling**

**P640**

**Pharmacological intervention of MRTF signaling reduces outgrowth initiation, progression, and bone metastasis of triple-negative breast cancer cells**  
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Myocardin-related transcription factors A and B (MRTF-A and MRTF-B) link actin cytoskeletal dynamics to activation of SRF (serum-response factor) and transcriptional control of a wide range of genes. In this study, we demonstrate that hormone receptor status determines the impact of MRTF expression on the clinical outcome in human breast cancer (BC). Specifically, higher expression of MRTF and/or its target genes correlate with prolonged and diminished survival (overall and/or relapse-free) of luminal and basal-like/triple-negative (TN) BC patients, respectively. Consistent with these clinical correlation data, knockdown and overexpression studies provide evidence for MRTF’s ability to promote single-cell outgrowth competency of TNBC cells in 3D culture *in vitro*. Accordingly, treatment with pharmacological inhibitor of MRTF/SRF signaling (CCG-1423 or its analog) retards single cell outgrowth as well as progression of established outgrowth of TNBC cells in 3D culture *in vitro*. Global transcriptome analyses of 3D cultures of TNBC cells show that CCG-1423 treatment impacts multiple biological pathways that are relevant for metastatic growth of cancer cells including actin cytoskeletal and integrin signaling, cell proliferation/survival, metabolism, angiogenesis, and immune cell-regulatory events. Finally, we demonstrate that CCG-1423 dramatically suppresses bone metastasis of TNBC cells *in vivo*, a finding that is consistent with our *in vitro* evidence for CCG-1423-induced alteration in the expressions of several bone metastasis-related genes. Based on these data, we propose that pharmacological intervention of MRTF signaling may be a promising strategy to prevent and/or diminish bone metastasis in TNBC.

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**Regulation of Transcription Factor Brain-2 (BRN-2/ Oct-7/ N-Oct3) by Integrin α3β1 Affects Breast Cancer Cell Invasion**  

The tumor microenvironment plays a critical role in regulating gene expression within tumor cells that drives malignant progression and invasion; however, roles for integrins in this process remain underexplored. The laminin-binding integrin, α3β1, is a heterodimeric cell surface protein that promotes tumor growth and metastasis in murine models of breast cancer, and our lab and others have demonstrated a role for α3β1 in promoting breast cancer cell invasion. Here we show using the *in vivo* tail vein experimental metastasis assay that integrin α3β1 promotes lung colonization of human triple-negative breast cancer cells (MDA-MB-231), consistent with other reports. To explore mechanisms of α3β1-mediated effects on cell invasion, we mined data from our published microarray analysis in which we compared the transcriptomes of MDA-MB-231 cells that express either non-targeting shRNA (control) or α3-targeting shRNA (α3-knockdown). We showed that knockdown of α3 leads to suppression of the mRNA encoding the transcription factor, Brain-2 (BRN-2). BRN-2 is a master regulator of the invasive phenotype in melanoma; however its role in breast cancer is unknown. **We hypothesize that integrin α3β1-dependent regulation of BRN-2 expression promotes invasion and lung...**
colonization of breast cancer cells. Here we confirm that RNAi-mediated targeting of α3 in MDA-MB-231 cells leads to reduced BRN-2 mRNA and protein levels. Additionally, we show reduced BRN-2 promoter reporter activity in α3-knockdown cells, indicating α3β1-dependent transcriptional regulation of BRN-2. Preliminary evidence indicates that α3β1 regulates BRN-2 expression by the PI3K/Akt signaling pathway. Further, we demonstrate a pro-invasive role for BRN-2 in MDA-MB-231 cells using RNAi-mediated knockdown of BRN-2 followed by Matrigel Transwell invasion assays. In summary, our work confirms a role for α3β1 in lung colonization of human triple-negative breast cancer cells in vivo, identifies a pro-invasive role for BRN-2 in breast cancer cells in vitro, and identifies a novel role for integrin α3β1 in the regulation of BRN-2, revealing a potential mechanism of gene regulation that affects breast cancer cell invasion.

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Beta-pix Facilitates Colorectal Cancer Invasion through Interaction with Dynamin2.
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The dynamics of plasma membrane by cytoskeleton rearrangements is required for cancer cell migration and invasion. In this study, we demonstrate that upregulated βPix, a guanine nucleotide exchange factor for Rac1, promotes migration and invasion of colorectal cancer (CRC) cells via interaction of Dynamin2. Affinity chromatography analysis and pull-down assay with βPix-SH3 domain revealed that the Dynamin2 (Dyn2), a large GTPase, interacts with βPix via its proline rich domain (PRD) at C-terminus. Interaction of βPix-Dyn2 occurred at the leading edge of plasma membrane, which in turn, facilitated Rac1 activation, resulting in the induction of dynamic membrane ruffling. We also found that the phosphorylation of tyrosine residue at 422 position of βPix by Src kinase was critical for βPix/Dyn2 complex formation. Interestingly, application of SH3 antibodies conjugating with gold nanoparticle for targeting intracellular βPix disrupts βPix/Dyn2 complex, resulting in inhibited cell invasion. Taken together, our study elucidates that Src-βPix-Dyn2 axis is essential for CRC cell invasion. Furthermore, the development of inhibitors disrupting the βPix/Dyn2 complex would be a useful therapeutic strategy for CRC treatment.

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Identification and characterization of mechanisms underlying formation of TSIPs, new collective and malignant intermediates mediating the dissemination of colorectal carcinomas.
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Colorectal cancer (CRC) is the second deadliest form of cancer worldwide, most of these deaths are due to metastases. To date we know that tumor cells use two main strategies of invasion: EMT and collective invasion. The first one is the most studied way of dissemination but there is still a lack of clinical translation at prognosis or therapeutic level. In the last years our team showed that, in peritoneal effusions from patients with aggressive and mucinous form of CRC, spherical clusters of hundreds of cells displaying an inverted apicobasolateral polarity are major invasive tumoral intermediates. We
called them TSIPs for Tumor Spheres with Inverted Polarity. Using cell line models recapitulating epithelial properties and molecular identity of the CRC of interest, my PhD project aims at deciphering the mechanisms underlying TSIPs formation. Preliminary results showed that TSIPs formation happens in serrated precursors of mucinous CRCs and preferentially in a TGFB signaling downregulated context. Our main hypothesis is a process resembling a “collective apical budding”, with several steps leading to emergence of a cluster of cells from a monolayer on the apical side, a process which has never been described yet. Through live video-microscopy we observed deformation, bulging and TSIP release in patient sample at the beginning which we were able to recapitulate in our CRC cell line model. We confirmed via immunostainings the outer localization of apical markers, characteristic of TSIPs. We also could assess the presence of a supracellular cortex of acto-myosin engaged in the process, validated by tests with contractility drugs. We are showing a preferential distribution of proliferating cells and a specific organization in budding structures in comparison to the monolayer, leading us to a proliferation-division role. Thanks to lentiviral modifications we are also assessing the role of ERK signaling, known as a cell cycle but also contractility regulator. We finally address the impact of mucin regulation and production as TSIPs formation occurs in mucinous CRCs. By understanding the formation of TSIPs as a new invasive tumoral intermediate in CRCs we wish to enlarge knowledge about collective dissemination in order to link our results to clinical applications, thus improving therapeutic approaches.

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Lateral macrophage mitochondrial transfer functions as a signaling source in cancer cells

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Unconventional modes of cellular communication such as the sharing of cellular components can have large impacts in the context of homeostasis and disease. Previously, we discovered that macrophages transfer cytoplasm to melanoma cells in vivo, with 70% of cytoplasm-receiving melanoma cells disseminating from the primary transplantation site. These data suggest macrophages transfer a “package” of molecules that influence recipient cell behavior. However, central questions remain: 1) what is identity of the transferred molecules? and 2) how do these molecules influence cell behavior? Here, we report that cell contact-dependent mitochondrial transfer occurs from macrophages to cancer cells, and recipient cancer cells exhibit an increased proliferative index. By genetically labeling mitochondria in primary human macrophages and breast cancer cells, we found that donated mitochondria do not incorporate into the host mitochondrial network, but instead persist as a separate, intact population. Interestingly, with the use of live dyes and a genetically encoded biosensor, we found that transferred mitochondria have reduced membrane potential and accumulate high levels of reactive oxygen species (ROS). Furthermore, with the use of a mitochondrial localized antioxidant we have shown that we can inhibit proliferation in recipient cancer cells. Taken together, these data suggest that the transferred mitochondria are serving as a signaling source rather than functioning as an energy-producing organelle. In future studies, we will address sufficiency by introducing purified mitochondria into cancer cells via microinjection and focus on identifying the ROS-responsive signaling pathway(s) underlying the proliferative response. Collectively, these studies will help to elucidate how donated molecules can lead to functional changes in recipient cells and define a type of cellular communication that contributes to disease progression.
An indoline derivative induces apoptosis in mid and late stage colorectal cancer cell lines

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Cancer is a group of diseases, characterized by abnormal division of cells. It is considered to be one of the most serious health problems worldwide. In South Africa, CRC is the fourth most common and the sixth leading cause of cancer mortality in South Africa. As an over-expression of pro-apoptotic genes and under expression of anti-apoptotic genes is responsible for carcinogenesis, the aim of this study was to evaluate the potential pro-apoptotic effects of a synthesized Indoline derivative (HNPMPI) on the HT29 (stage 2) and DLD1 (stage 3) colorectal adenocarcinoma cell lines. Based on the IC50 concentration, the in vitro effects of HNPMPI treatment on apoptosis were assessed, using a focused human apoptosis protein array (Annexin V), to identify differentially regulated proteins; and from this analysis, gene expression levels of selected genes were analyzed using RT-PCR. Further, the subcellular localization of key proteins were determined using confocal microscopy. The Annexin V profiling demonstrated that HNPMPI more effectively induced apoptosis in HT29 cells compared to the DLD1 cell line. The protein profiler assay revealed differential expression of apoptotic proteins, demonstrating activation of the extrinsic apoptotic pathway in the HT29 cell line; and triggering of the Caspase mediated intrinsic apoptotic pathway in DLD1 cells, after treatment. Specifically, post HNPMPI treatment, the pro-apoptotic genes BAD, BAX and p53 decreased in DLD1 cells; while in the HT29 cell line, BAX and p53 decreased and BAD levels increased. The anti-apoptotic gene, CASP-3 increased in both cell lines; while Bcl-2 levels were constant in the HT29 cells, but decreased in DLD1 cells. In support of this, immunofluorescence confirmed that the intracellular protein expression of BCL-2 and P53 reflected the results obtained from the protein array following drug treatment. In summary, the protein expression results obtained here confirm that HNPMPI has promising pro-apoptotic properties acting to induce both the intrinsic and extrinsic pathways of cell death in a stage specific manner in the two colon cancer cell lines.

The large GTPase, GBP-2, regulates Rho family GTPases to inhibit migration and invadosome formation in breast cancer cells.

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Breast cancer is the most common cancer in women. Despite advances in early detection and treatment, it is predicted that over 40,000 women will die of breast cancer in 2019. To lower this number, more information about the molecular players in breast cancer are needed. Guanylate-Binding Protein-2 has been correlated with better prognosis in breast cancer. We show that GBP-2 expression correlates with longer recurrence-free survival, longer overall survival, and longer time of distance free metastasis for breast cancers of all types. In this study, we asked if the expression of GBP-2 in breast cancer merely provided a biomarker for improved prognosis or whether it actually contributed to improving outcome. To answer this, the 4T1 model of murine breast cancer was used. 4T1 cells themselves are highly
aggressive and highly metastatic, while 67NR cells, isolated from the same tumor, do not leave the primary site. The expression of GBP-2 was examined in the two cell lines and found to be inversely correlated with aggressiveness/metastasis. Proliferation, migration, and invadosome formation were analyzed after altering the expression levels of GBP-2 in the cell lines. Our experiments show that GBP-2 does not alter the proliferation of these cells but inhibits migration and invadosome formation downstream of regulation of Rho GTPases. Together these data demonstrate that GBP-2 is responsible for cell autonomous activities that make breast cancer cells less aggressive.

Tuesday, December 15, 2020, 12:00 pm

Actin Dynamics in Cell Locomotion

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Melanoma A375 cells under non-adhesive confinement produces mesenchymal-to-Leader bleb transition and Leader Bleb-based migration to be mediated by Filamin crosslinking protein

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Cancer cells migrating in confined 3D microenvironments mimicking the tissue microenvironment exhibit plasticity of migration modes. Under high confinement, actomyosin contractility and low adhesion, intracellular pressure drives the formation of a large bleb, that mediates mesenchymal-to-leader bleb formation and rapid persistent migration ‘leader bleb-based migration’ (LBBM), characterized by an unusual, highly polarized cell morphology with a long (~20 um) sausage-shaped bleb that points in the direction of movement, separated from a smaller (~10-15 um) spherical cell body by a contractile neck. Actomyosin assembly and retrograde flow along the bleb coupled to non-specific friction with the microenvironment drives mesenchymal-to-leader bleb transition and LBBM. Fundamental organization of organelles nor actin regulatory protein’s role in mediating this unusual mode of motility is known. We demonstrate localization of organelle markers and actin-associated proteins in metastatic human A375 melanoma cells undergoing LBBM. We expressed fluorescent fusion proteins in cells confined to a low adhered 3um space under a polydimethylsiloxane (PDMS) pad and imaged by time-lapse spinning-disk confocal microscopy describing spatial distribution within the cell body and leader bleb, as well as cell motility parameters. We also measure mechanical dynamics of loosely adhered cells using Atomic Force Microscopy (AFM). Most cytoskeleton and membranous organelles examined are localized in the cell body; however, microtubules as well as the Golgi and ER also extend into the leader bleb and the nucleus and centrosome translocate between body and bleb. We found that actin nucleators localizes towards the leader bleb tip, while crosslinkers are found exclusively in the bleb. Analysis of morphology and motility parameters in cells revealed that α-Actinin-1 and Filamin-A promotes leader bleb formation, speed and directionality. We then tested the hypothesis that Filamin-A is an effector to modulate actin cortex mechanics, thereby mediating bleb-based formation. We find that using AFM, Filamin-A influences cellular mechanics through cortical tension and intracellular pressure. Our study provides the first description of the cellular “anatomy” during mesenchymal-to-leader bleb transition and LBBM, suggesting that actin crosslinkers are important in
regulating leader bleb size, speed, and directionality under low adhesive confinement and help mediate cellular mechanics.

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**VCA accelerates both the nucleation of actin filaments by Arp2/3-SPIN90 complex and the recycling of SPIN90s**

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Arp2/3 is one of the most important actin nucleators. Activated by the VCA domains, which present in both WASP and WAVE family proteins, Arp2/3 attaches to the side of F-actin and generates actin branches; activated by SPIN90, Arp2/3 nucelates de novo linear filaments. Previous results have highlighted the competition between VCA and SPIN90 in shaping the architecture of cortical actin networks (ref. Cao et al, NCB, 2020). However, structural results (ref. Luan et al, EMBO J, 2018) suggested that VCA and SPIN90 can bind simultaneously on an Arp2/3 complex, suggesting a more complex synergy/competition between the two proteins. To test this hypothesis, we used fluorescence microscopy combined with microfluidics, to probe the nucleation of surface-anchored SPIN90-Arp2/3 complex. In the presence of VCA, the nucleation rate mediated by SPIN90-Arp2/3 is increased 2.5 fold. Surprisingly, we also observed that the good affinity binding of VCA greatly accelerates the dissociation of the SPIN90-Arp2/3 complex. These two observations reveal that VCA speeds up the nucleation efficiency of de novo filaments by the SPIN90 and Arp2/3, up to ~4 fold, a mechanism that might be key to the rapid initialisation of cortical actin networks in cells. Our results shed light on the potential synergy that can arise between nucleation promoting factors to control the shape and dynamics of actin networks.

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**Dual regulation of the actin cytoskeleton by CARMIL-GAP**

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CARMIL (Capping protein Arp2/3 Myosin I Linker) proteins are ~1100 residue, multi-domain scaffold proteins expressed from protozoa to man that regulate actin cytoskeletal dynamics by regulating the activity of Capping Protein (CP). Evidence is growing that CARMILs also regulate signal transduction pathways, as several interact with Trio, a guanine nucleotide exchange factor for multiple Rho-related GTPases, and T cells lacking CARMIL-2 exhibit a profound signaling defect. We showed previously that *Dictyostelium* CARMIL promotes several actin-dependent processes by binding to and regulating CP. Here we characterize the function of CARMIL-GAP, a second *Dictyostelium* CARMIL that contains a ~130 residue insert that, by homology, is a GTPase activating (GAP) domain for Rho-related GTPases. Consistently, we show that this putative GAP domain binds the *Dictyostelium* Rac isoform Rac 1A and accelerates its rate of GTP hydrolysis. CARMIL-GAP concentrates with F-actin in phagocytic cups and at the leading edge of migrating cells, and cells devoid of CARMIL-GAP exhibit pronounced defects in phagocytosis and chemotactic aggregation. Importantly, these defects are specific to the loss of CARMIL-
GAP, as its expression in CARMIL-GAP null cells results in full rescue. Finally, the rescue of CARMIL-GAP null cells with versions of CARMIL-GAP that lack either GAP activity or the ability to regulate CP show that while both activities contribute significantly to CARMIL-GAP function, the GAP activity plays the bigger role. Together, our results add to the growing evidence that CARMIL proteins regulate actin dynamics by regulating signaling molecules as well as CP, and that the cycling of Rho GTPases between their GTP-bound and GDP-bound states is required to drive biological processes.

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Regulation of the cortical actomyosin cytoskeleton by ADF/cofilin is required for the bleb-based migration of cancer cells

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The cortical actomyosin cytoskeleton lies directly underneath the plasma membrane of all cells. In cancer cells, a high level of cortical actomyosin contractility is correlated with invasiveness. Recently, it was discovered that cancer cells can undergo fast amoebooid or Leader Bleb-Based Migration (LBBM), which requires the formation of a large and stable bleb for migration. Contained within all leader blebs, is a fast cortical actomyosin flow driving the cell forward, however the fundamental mechanisms required to maintain contractility and actin flow in leader blebs is not understood. Here, we hypothesize that actin severing factors, such as the ADF/cofilin family and gelsolin, are essential to maintain both contractility and actin flow for LBBM. Using RNAi in melanoma A375-M2 cells and a flow cytometry-based method for measuring F-actin, we find that co-depleting ADF and cofilin and not gelsolin led to a large increase in the level of F-actin, suggesting that ADF and cofilin together regulate actin in these cells. Moreover, using barbed-end assays and high-resolution imaging, RNAi of ADF and cofilin increased the number of cortical, polymerization competent, barbed-ends. Therefore, severing by these proteins appears to promote cortical actin turnover in melanoma A375-M2 cells. Furthermore, actin severing has been shown to promote contractility through the regulation of actin architecture. In line with this concept, RNAi of ADF and cofilin significantly increased cell deformability, as determined by a gel sandwich approach. As LBBM is stimulated by cell confinement, we next used a PDMS slab-based approach, which uses micron-sized beads for the precise control of cell confinement, to evaluate the role of ADF/cofilin in regulating cortical actin dynamics in blebbing cells. Ratio imaging of EGFP-cofilin and mScarlet revealed cofilin to be enriched within leader blebs, whereas RNAi of ADF and cofilin reduced bleb sizes and the frequency of motile cells. Strikingly, cells without ADF/cofilin had exceptionally long necks separating blebs and the cell body. Many blebs failed to retract and exhibited slower actin turnover and cortical actin flow in the absence of ADF and cofilin. Importantly, myosin accumulated along the long bleb necks which was coupled with actin accumulation. Therefore, actin severing by these proteins appears to be critical to the function of the contractile bleb neck. Collectively, our data identifies ADF and cofilin as cortical actin remodeling factors required for the amoebooid migration of metastatic cancer cells.
Nucleotide coding sequence mediated translation dynamics of β- and γ-actin regulates cell migration

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β- and γ-cytoplasmic actins are ubiquitously expressed in every cell type and are nearly identical at the amino acid level, but play vastly different roles in vivo. Their essential role in embryogenesis is defined largely by the nucleotide sequence of their genes, rather than their amino acid sequence, however it is unclear which gene elements underlie this effect. Here we address the specific role of the coding sequence in β- and γ-cytoplasmic actins’ intracellular function, using stable cell lines with exogenously expressed actin isoforms and their “codon-switched” variants. When targeted to the cell periphery using the β-actin 3’UTR, β-actin and γ-actin have differential effects on cell migration. These effects are directly coding sequence dependent. Single molecule measurements of actin isoform translation, combined with fluorescence recovery after photobleaching, demonstrate a pronounced difference in β- and γ-actin’s translation elongation rate and in their turnover at the focal adhesions. Computational simulations predict that lower actin synthesis rate near the focal adhesion results in shorter actin bundles, leading to impairments in actin bundle formation and cell anchoring to the substrate during migration. Our results demonstrate that coding sequence-mediated actin translation plays a key role in cell migration in actin isoform-specific manner.

Actin Redox Balance Regulates Filament Disassembly

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Introduction: The assembly and disassembly of actin filaments (F-actin) are tightly controlled processes with critical implications for cell physiology. While many actin binding proteins, such as the ubiquitous ADF/cofilin, have been identified to directly interact and disassemble filaments, a complementary mechanism has been recently identified: actin oxidation. We recently showed that F-actin disassembly controls the timing of cytokinetic abscission, the terminal step of cell division, and that the dysregulation of actin dynamics can have dramatic consequences such as tetraploidy (Frémont et al. Nature Comm. 2017, Bai et al. PNAS 2020). In the cytokinetic bridge, we also revealed that F-actin redox balance is mediated by two enzymes, MICAL1 and MsrB2, which oxidizes and reduces key methionines on actin (Met44 and Met47), respectively. Question: It is unclear whether MICAL1 and MsrB2 can modify and possibly compete for the same pools of actin (F-actin vs. monomers). In addition, a synergy between ADF/Cofilin and MICAL1 for F-actin severing has been previously shown (Grintsevitch et al. NCB 2016), but the molecular mechanism is unknown and the interplay between F-actin oxidation and other regulatory proteins such as Tropomyosin has not been investigated yet. Method: To explore these questions, we performed in vitro experiments on single actin filaments, anchored within microfluidic chambers designed in the lab, and visualized with TIRF microscopy. Results: We first showed that MICAL1 selectively oxidizes F-actin but not monomeric actin (G-actin). In contrast, MsrB2 only reduces G-actin, but not oxidized actin filaments. We also found that Actin oxidation accelerates filament
depolymerisation 10-fold from both barbed and pointed ends. However, contrary to what was initially reported, oxidized F-actin does not spontaneously fragment. ADF/cofilin is a family of proteins at the centre of virtually all F-actin disassembly machineries. We found that F-actin oxidation boosts ADF/cofilin activity by accelerating both its recruitment to F-actin (nucleation and elongation of ADF/cofilin domains) and the severing rate at the border of ADF/cofilin domains. Furthermore, we found that oxidized F-actin gets rapidly disassembled even when they are protected by Tropomyosin or when ADF/cofilin is inhibited by phosphorylation. **Conclusion:** We conclude that F-actin oxidation is a powerful mechanism to regulate filament stability. Oxidized filaments are indeed quickly disassembled in circumstances that would otherwise be of little consequences: when ADF/cofilin is at very low concentration or inhibited by phosphorylation, and when filaments are protected by Tropomyosin.

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**Stabilization and motility mechanisms of blebs in confined cells**

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Previously associated with apoptosis, blebs have arisen in the past decade as **important structures for amoeboid cell migration**, particularly for cancer cells. In recent years, a few independent studies have reported large and stable blebs in cells under non-adhesive confinement. This universal switch to bleb-based migration has been found in amoeba, choanoflagellates, immortalized cell lines and primary cultures. Unlike previous blebs described, they are able to overcome retraction and stabilize a constant flow. Stable blebs are a new type of cellular structures that amoeboid cells use to migrate, analogous to filopodia or lamellipodia for mesenchymal cells. In a single cell, multiple blebs form and compete against each other, so that eventually a single bleb drives the migration. Thus, it is important to know how single blebs are stabilized to understand how single-bleb amoeboid cells polarize. More generally, stable actomyosin flows constitute the basis of migration. **The goal of this project is to study bleb morphogenesis and bleb stabilization in confined cells,** using microfluidics to control the confinement height, coating, and topography. The first part of the project describes the blebs forming as an immediate response of cells to confinement and what differentiates it from a classical retracting bleb. The second part focuses on the mechanism leading to the establishment of a retrograde flow. Based on the results, we propose that bleb stabilization depends on 1) the depletion of actin by myosin contractility and 2) the particular actin filament arrangement at the bleb tip caused by the membrane topology of a confined cell. I completed this work with advanced imaging (highNA TIRF, polarization microscopy) which allowed observation of single actin filaments and tagged cytoskeleton-associated molecules at the bleb tip, under different perturbations. This unique set of observations allowed to complete a model for the stabilization of motile blebs, with conclusions that can be generally applied to any flowing actomyosin cortex. We identified **three cortex regimes in blebs based on the relative actin and myosin densities:** 1) Assembling loose cortex: localized at the tip, composed of single filaments poorly attached to the membrane. If this region is lost, the bleb retracts. 2) Crosslinking cortex: actin filaments and fibers bind together to form a network which gradually gets denser and reticulated but do not contract (this region is devoid of Myosin II motors). 3) Contracting cortex: towards the base of the bleb. Myosin-II starts to get enriched contracting the dense actin network, driving the entire retrograde actin flow up to the tip of the bleb, generating new actin free regions at the tip and pressurizing the bleb, leading to membrane protrusion at the very front.
Cadherins and Cell-Cell Interactions

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Mapping Transmembrane Binding Partners For E-cadherin Ectodomains.
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Transmembrane proteins play essential roles in coupling cells and in sensing mechanical and biochemical signals from the environment. However, it is extremely challenging to identify membrane protein interactions using traditional methods like affinity pulldown. While proximity-tagging methods have been employed to map membrane protein interactomes, these assays merely report on protein proximity and testing direct interactions requires use of a complementary biophysical tool. However, the number of ‘hits’ generated by a typical proximity-tagging assay are enormous (hundreds of candidate proteins), which limits the number of binding interactions that can be directly tested. We therefore developed a highly targeted proximity screening method by tagging both the extracellular and cytoplasmic regions of a transmembrane ‘bait’ protein with BioID biotin ligase. Since transmembrane binding partners are positioned in close proximity to both the bait’s extracellular and cytoplasmic domains, they are biotinylated in both regions. We then directly tested binding interactions between proximal proteins and the bait using single molecule Atomic Force Microscopy. Using this approach, we identified several previously unknown direct interactions in epithelial cells, between transmembrane proteins and the extracellular regions of E-cadherin, an essential cell-cell adhesion protein. We demonstrate that besides forming homophilic bonds, the extracellular region of E-cadherin also binds to the ectodomains of the desmosomal proteins desmoglein-2 and desmocollin-3, the focal adhesion protein integrin-α2β1, and the receptor tyrosine kinase ligand ephrin-B1. Our results demonstrate that cadherin ectodomains do not merely engage in homophilic binding, but can also heterophilically interact with a range of junctional proteins.

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Differential spatio-temporal expression of type I and type II cadherins during development of the central nervous system associated with neuromeric segmentation and formation of brain nuclei
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Type I and type II classical cadherins comprise a family of cell adhesion molecules that regulate specific cell sorting and formation of tissue boundaries by forming homo and heterophilic bonds with varying affinities. Factors that affect cadherin-mediated cell adhesion include cadherin binding affinity and expression level. This study examines the expression of cadherins type I (Cdh1, Cdh2, Cdh3 and Cdh4), type II (Cdh6, Cdh7, Cdh8, Cdh9, Cdh10, Cdh11, Cdh12, Cdh18, Cdh20 and Cdh24), and the atypical Cdh13 during distinct morphogenetic events in the developing mouse central nervous system (CNS). Cadherin mRNA expression values from embryonic day (E) 11.5 to postnatal day (P) 56 mouse were obtained from in situ hybridization experiments carried out at the Allen Brain Institute and available at the Allen Developing Mouse Brain Atlas. Classical cadherins expression levels peak at the end of embryonic development (E18.5) and early postnatal life (P4); however, each cadherin shows a distinct spatiotemporal expression pattern. Analyses of the relative adhesive strength of each neuromeric
segment of the neural tube show differences in relative adhesion along the rostral-caudal axis driven by changes in the expression Cdh2 and type II cadherins, suggesting that variances in cadherin-mediated adhesion contributes to neuromeric segmentation. During embryonic development (E13.5 and E15.5) of the subpallium, Cdh2, Cdh13, Cdh8 and Cdh11 expression is higher in anatomic structures derived from the superficial stratum as compared to the periventricular and intermediate strata. By P56, Cdh6 and Cdh9 expression levels increase across the subpallium. At P56, Cdh6, Cdh7, Cdh8, Cdh10 and Cdh12 are abundant in the intermediate layers (layers 5 to 3) of the cerebral cortex (dorsal pallium), while Cdh2, Cdh13 and Cdh11 show a graded expression from deeper (layer 6) to superficial layers (layer 1), and Cdh9, Cdh18 and Cdh24 tend to be more profuse in the deeper layers. This analysis shows a highly dynamic spatial and temporal expression pattern of type I and type II cadherins across the developing CNS, suggesting that classical cadherins expression is regulated locally to specify molecular identity and adhesive properties to each neuronal group necessary for the formation of brain nuclei, neuronal layers and synaptic connections.

Retromer-mediated Dsg1 Endosomal Trafficking is Necessary for Keratinocyte Differentiation and Stratification

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The epidermis is a complex stratified epithelial tissue that regenerates through proliferation of basal cells that eventually exit the cell cycle, transit into the superficial layers, and undergo a highly orchestrated process of differentiation to create the skin barrier. The desmosomal cadherin, desmoglein-1 (Dsg1) is first expressed as keratinocytes commit to differentiate. In addition to its critical role in intercellular adhesion, keratinocyte stratification and differentiation requires Dsg1’s proper expression and localization. The importance of Dsg1’s proper localization is reflected by the existence of the human disorder Severe dermatitis, multiple Allergies, and Metabolic wasting (SAM syndrome), caused by loss of function mutations in Dsg1 that can affect the proper trafficking and accumulation at the cell surface through mutations in the protein’s N-terminal signal sequence. Our previous data showed that the dynein light chain, Tctex-1, was required to properly position Dsg1 on the plasma membrane. Considering the observation that dynein plays a role in endosome-mediated recycling of transmembrane proteins, we hypothesized that endosome mediated trafficking is necessary for Dsg1 plasma membrane localization. Consistent with our hypothesis, inhibition of dynein or endosomal trafficking disrupts Dsg1 plasma membrane localization. Using a BioID interaction screen, we identified VPS35, an integral component of the endosomal trafficking complex called the retromer, as a putative Dsg1 interacting protein. Depletion of VPS35 or VPS29, another essential retromer component, disrupts Dsg1 plasma membrane localization, mistargeting Dsg1 to the lysosome and resulting in decreased protein levels of Dsg1, but not Dsg3 or E-cadherin. Loss of retromer function also disrupted cortical actin organization and consequent Dsg1-mediated keratinocyte stratification, consistent with the requirement of retromer-mediated trafficking for Dsg1 function. A pharmacological chaperone which enhances retromer function (R55) is sufficient to enrich wild-type and a SAM syndrome-associated Dsg1
mutant at the plasma membrane. In 3D organotypic epidermal cultures, VPS35 depleted cultures displayed signs of aberrant differentiation and morphogenesis, while R55-treated cultures exhibited signs of enhanced epidermal differentiation. Altogether, our data support the retromer’s role in trafficking Dsg1 to the plasma membrane to promote cortical actin remodeling and subsequent keratinocyte differentiation and morphogenesis.

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Irf6 regulates the delivery of E-cadherin to the cytoplasmic membrane
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IRF6 regulates the delivery of E-cadherin to the cytoplasmic membrane Angelo Antiguas, Martine Dunnwald Department of Anatomy and Cell Biology Interferon regulatory factor 6 (IRF6) is a transcription factor that belongs to the IRF family of transcription factors. Mice lacking Irf6 lack a functional epidermal barrier, partially because of altered intercellular junctional complexes in the upper layers of the epidermis. Therefore, we hypothesize that Irf6 is required for proper intercellular adhesions formation. We used wildtype (WT) and Irf6 KD keratinocytes in vitro to test our hypothesis. Immunodetection of intercellular adhesion proteins showed an altered subcellular localization of all the adhesion proteins at the plasma membrane upon adhesion engagement in Irf6 KD compared to WT keratinocytes. This phenotype was accompanied by a loss of mechanical integrity and a delay in the formation of new AJ in Irf6 KD keratinocytes. Following biotinylation assays, we demonstrated that levels of E-cadherin and Occludin were decreased at the plasma membrane in Irf6 KD keratinocytes, while their overall level in the cells was unchanged. We further showed a similar rate of endocytosis between WT and Irf6 KD keratinocytes. To determine if Irf6 regulates trafficking of E-cadherin, we measured the recovery of E-cadherin-GFP fluorescence after photobleaching. Our results demonstrated that both, the time needed to recover 50% of the initial fluorescent intensity, and the recovered fraction, were significantly decreased in Irf6 KD compared to WT keratinocytes, suggesting that the delivery of E-cadherin to the plasma membrane is dependent on Irf6. Follow up internalization assay demonstrated that Irf6 was required for the recycling of E-cadherin to the plasma membrane. Overall, our data suggest a role for Irf6 in the recycling of E-cadherin to the plasma membrane. As E-cadherin is essential for adherens junction assembly, which themselves affect other intercellular junctions, we propose that Irf6 is a critical factor for junctional complexes formation.

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E-cadherin: Unexpected actor of invadopodia formation in pancreatic cancer
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Extracellular matrix remodelling is known as one of the causes of tumoral invasion and metastasis. Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest cancers in the world particularly due to its late diagnosis and its resistance to chemotherapeutic agents. The complexity of this pathology is implemented by a dense desmoplastic stroma with Type I collagen as a major component, an expansive tissue invasion and early metastasis. Alterations in the expression of cell adhesion molecules such as cadherins have been reported in PDAC. Yet, how these changes contribute to tumour progression is
poorly understood. Our previous study demonstrates the different implications of E-cadherin and P-cadherin in pancreatic tumour cells invasion. In this study, we aim to decipher the function of E-cadherin in pancreatic tumour matrix remodelling through invasive structures, called invadopodia. By performing invadopodia assays, we demonstrate a functional role of E-cadherin in invadopodia formation. Indeed, E-cadherin depletion by shRNA strategy in pancreatic tumour cell line (BxPC-3) reduce the number of cells positive for invadopodia. This unexpected observation is confirmed using E-cadherin synthetic inhibitors. Even more surprisingly, a pool of E-cadherin was detected by confocal microscopy in colocalization with invadopodia markers (cortactin, Tks5; ...) into the actin protrusion. These data show an unknown localization of E-cadherin and suggest a physical and/or functional interaction of the E-cadherin with others invadopodia components. Immunoprecipitation and proximity ligation assay (PLA) confirmed the physical interaction between E-cadherin and MT1-MMP into invadopodia. By siRNA strategy, we show that E-cadherin recycling occurs in these invasive structures, as the MT1-MMP, though 2 different pathways: Rab-7 and Rab-11 dependant. Moreover, proteomic analysis of BxPC-3 cells show that E-cadherin depletion promotes a down-regulation of the actin nucleation pathway through Arp2/3 complex, which can be explained by the down-expression of all Arp2/3 complex subunits. As the key component of branched actin structure, Arp2/3 complex loss prevent invadopodia formation. Together, our studies show a new E-cadherin function in tumour development. In pancreatic tumour cell, E-cadherin will take part in invadopodia formation and could be used to evaluate invasive properties of tumours cells patients.

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Elucidating tumor heterogeneity via explore Ca\textsuperscript{2+}-adhesion interactions in ovarian clear cell carcinoma cell
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Elucidating tumor heterogeneity via explore Ca\textsuperscript{2+}-adhesion interactions in ovarian clear cell carcinoma cell

Abstract Patients with ovarian clear cell carcinoma (OCCC) usually have poor prognosis with limited treatments, probably due to the heterogeneity between cancer cells with the tumor. Consistent with this recent investigation revealed important but differential effect of Ca\textsuperscript{2+} on the migration of endothelial and cancer cells, probably related to the complex Ca\textsuperscript{2+}-adhesion interactions. Moreover, data from TCGA database showed potential contribution of Ca\textsuperscript{2+}-regulating molecule ORAI1 to the progression of ovanrian cancer, and inverse correlation between ORAI1 expression and adhesion molecules in tumor specimens. We thus postulated that Ca\textsuperscript{2+}-adhesion interactions were attributed to the heterogeneity of OCCC and its prognosis. To confirm this, we selected two different OCCC cell lines JHOC-9 (epithelial-like) and OVTOKO (mesenchymal-like) for random collective migration assays. Inhibitor BTP2 (0, 0.25, 0.5, 1, 2, 4μM) and ROCK inhibitor Y27632 (0, 1.25, 2.5, 5, 10μM) were recruited to alter Ca\textsuperscript{2+} and adhesion signaling of these cell lines in random collective migration assays. Western blotting were also adopted to observe the expression of Ca\textsuperscript{2+}-regulating molecules and adhesion molecules. As predicted JHOC-9 showed slower migration but better cell-cell coordination than OVTOKO in random collective migration assays. Moreover, increased the motility of JHOC-9 but reduced the motility of OVTOKO. Together with the discovery that ORAI1 expression in JHOC-9 was higher than OVTOKO by western blotting, our preliminary results suggest integrated interaction between Ca\textsuperscript{2+} and adhesion signaling controlling the
migration of OCCC cells. In the future, we will further clarify the mechanism underlying such Ca\textsuperscript{2+}-adhesion interaction, and develop corresponding strategies to target tumor heterogeneity in OCCC.

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**Mmp-7 increases migration by decreasing intercellular E-cadherin and F-actin localization in prostate tumor spheroids formed by perlecan/HSPG2**

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Polarized epithelium is maintained by cell-cell interactions via cadherins and other cell adhesion molecules (CAMs). Interactions are stabilized further by cell-matrix interactions on the basement membrane where perlecan/HSPG2 is a major component that controls signaling in resting tissues with unperturbed matrices. Proteolytic cleavage of perlecan increases cell-matrix interactions and dysregulates cell signaling, permitting migration. Previous studies showed perlecan domain IV-3 (DmIV-3) drives cell cohesion and, when digested with matrix metalloproteinase-7 (MMP-7), drives cell dyscohesion. In vivo, prostate cancer (PCa) patients with bone metastases have circulating DmIV fragments, with negative correlation between MMP-7 staining and loss of perlecan in tissue samples. MMP-7 can cleave cadherins and other CAMs disrupting cell-cell adhesions. Also, DmIV-3 fragments generated by MMP-7 cleavage may further induce cell dyscohesion by disrupting interactions between CAMs. **Methods:** To evaluate the impact of MMP-7 and perlecan fragments on cluster dyscohesion and cell migration, uniformly sized PCa cell clusters were pre-formed using a microwell system. Pre-formed cell clusters were transferred to perlecan DmIV-3 (DmIV-3) or full length perlecan (FL pln) coated wells for 16-24 hours. Clusters were treated with MMP-7 alone or MMP-7 plus DmIV-3 fragments or MMP-7 plus FL pln fragments. For live cell imaging, cell tracking of migratory cells leaving the clusters was performed using Imaris software. **Results:** Pre-formed PCa cell clusters cultured in the presence of DmIV-3 cleaved by MMP-7 showed lower Pearson’s correlation values at cell boundaries in comparison to cell clusters treated with intact DmIV-3. Also, line scan analysis revealed E-cadherin and F-actin fluorescent signals were enriched and co-aligned in cell clusters treated with DmIV-3; this enrichment and co-alignment was reduced in the presence of DmIV-3 fragments and MMP-7. The number of tracks detected per cell cluster was highest in the presence of FL pln fragments plus MMP-7 along with a modest change in distribution of track displacement lengths of individual cells toward high values. **Conclusion:** Following patterns of dyscohesion of pre-formed PCa micro-tumors provides a good model to study dynamic changes in protein components involved in cell-cell interactions and quantitate cell migration patterns as they can occur in metastasis and circulating tumor cell formation. Actin reorganization promotes a migratory cell phenotype in PCa cell clusters treated with MMP-7 and DmIV-3 fragments. Future studies aim to identify DmIV3 fragment(s) positively associated with tumor dyscohesion that may play key roles in metastasis formation.
Cardiovascular, Exocrine, and Endocrine Systems

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**Macrophage-dependent iron homeostasis**

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Redox reactions are a core component of cellular processes, but as with most things in life, everything must be in moderation. Iron is the primary cofactor in redox chemistry and functions in many cellular activities, including proliferation. Excess iron is toxic to cells, therefore proliferative cells with high metabolic requirements for iron continuously acquire iron, rather than storing large quantities. Macrophages, a component of the immune system, are unique and can maintain large deposits of iron. The capability to store iron allows macrophages to use iron as part of the innate immune response. Macrophages can sequester and store iron, or secrete iron as needed, releasing it to the microenvironment. How does the cross talk between macrophages and the environment affect iron release and iron homeostasis of surrounding cells? To model these interactions, we use primary human blood monocyte-derived macrophages and highly proliferative and iron-dependent breast cancer cells. Our preliminary findings suggest that interactions with macrophages alter cellular iron import, export and iron storage in cancer cells. We found a reduction in tumor ferritin and a corresponding increase in tumor expression of transferrin receptor, when breast cancer cells were cultured with macrophages. Surprisingly, we have also identified a novel mechanism by which macrophages may transfer iron or iron associated proteins to cells through contact-mediated mechanisms, independent of established mechanisms of iron secretion. These data suggest that macrophages regulate iron homeostasis through direct transfer of components. Taken together, our work will provide insight into how cells with high iron requirements are affected by interactions with macrophages, and how this communication translates to cellular function and disease progression. Iron chelation is ongoing in clinical trials but understanding how iron homeostasis changes based on the microenvironment will be beneficial in determining the correct course of treatment and aid in discovery of novel drug targets.

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**Astrocytes integrate a circuit of neurovascular dysfunction during brain vascular malformations**

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Cerebral cavernous malformations (CCMs) are common neurovascular lesions caused by loss-of-function mutations in one of three genes, including *KRI1* (CCM1), *CCM2*, and *PDCD10* (CCM3), and generally regarded as an endothelial cell-autonomous disease. Here we report that proliferative astrocytes play a critical role in CCM pathogenesis by serving as a major source of VEGF during CCM lesion formation. An increase in astrocyte VEGF synthesis is driven by endothelial nitric oxide (NO) generated as a consequence of KLF2 and KLF4-dependent elevation of eNOS in CCM endothelium. The increased brain endothelial production of NO stabilizes HIF-1α in astrocytes, resulting in increased VEGF production and expression of a “hypoxic” program under normoxic conditions. We show that the upregulation of cyclooxygenase-2 (COX-2), a direct HIF-1α target gene and a known component of the hypoxic program,
contributes to the development of CCM lesions because the administration of a COX-2 inhibitor significantly prevents the progression of CCM lesions. Thus, non-cell-autonomous crosstalk between CCM endothelium and astrocytes propels vascular lesion development, and components of the hypoxic program represent potential therapeutic targets for CCMs.

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**Polyphenols Modulate Quiescence/Mobilization of Hematopoietic Stem Cell by Inhibiting Positive Regulation of CXCR4**

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Hematopoietic stem cell (HSC) remains quiescent in the bone marrow in homeostasis. In non-homeostatic or stress conditions such as infections, HSC increases in the circulation. The CXCL12 chemokine and its major receptor CXCR4 are essential for HSC retention in the bone marrow. CXCL12 secretion by bone marrow stromal cells induced by a stress and its release into the circulation are accompanied by the positive regulation of CXCR4 in HSC, inducing its recruitment to the periphery. Our goal was to investigate polyphenols effect on: 1) CXCL12; 2) regulation of CXCR4 on HSC and progenitor cells; 3) mature cells. Polyphenols from green tea (250mg/kg) was given once every 7 days orally (gavage) to C57BL/6 mice (n=6/group) challenged with i.p. LPS injection (100µg). Control group received vehicle only. After 1h and 24h, mice were sacrificed; PB and BM were collected for the analysis of: 1) CXCL12 levels in the bone marrow fluid and serum by ELISA; 2) % HSC (Lin−Sca1+cKit+) CXCR4+ and progenitor (Lin−cKit+) CXCR4+ cells by flow cytometry; 3) white blood cell (WBC) counts using hematology analyzer and % Lin+ cells (including: T and B cells, monocytes and granulocytes) by flow cytometer. LPS injection reduced CXCL12 in the bone marrow after 1h (p<0.005), whereas increased CXCL12 in the peripheral blood after 24h (p<0.001), leading to the recruitment and mobilization of HSC CXCR4+ and Lin−cKit+CXCR4+ cells into circulation (p<0.005). An increase of WBC number and Lin+ cells % was also observed (p<0.05). Treatment of LPS-injected mice with green tea polyphenols was able to mitigate LPS effect in the most immature cells, since it partially maintained CXCL12 levels in the bone marrow niche (p<0.05) and reduced HSC CXCR4+ and Lin−cKit+ CXCR4+ cells in peripheral blood (p<0.05), without affecting mature cells and circulating levels of CXCL12. CXCR4 is a multifunctional receptor coupled to G protein that is activated by its natural ligand, CXCL12. As a probable member of the LPS-sensitive complex, CXCR4 is involved in the production of pro-inflammatory cytokines and exhibits activity in chemotaxis. Interestingly, in a previous work, we observed that green tea reduced CXCR4 expression in immature leukemia CD34+ and cKit+ cells by decreasing reactive oxygen species and inhibiting HIF-1α activation (Cancer Lett. 2018;414:116). It appears therefore that green tea polyphenols effect in recruiting HSC is dependent on their anti-inflammatory action, possibly triggered by regulation of CXCR4 in the most immature cells, without affecting mature cells.
Perinatal exposure to bisphenol A affects the inflammatory profile of adult female prostate environment

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The prostate is present in females as a well-developed gland in several species, including humans, mice and bats. It is an active organ, which secretion has specialized functions on reproduction, promoting spermatozoa motility and survival in the female reproductive tract and vaginal lubrication. Prostate development starts during the embryonic susceptibility window period. This development process is modulated by endocrine mechanisms and may be affected by endocrine disruptors (ED), such as bisphenol A (BPA). These ED negatively impact the long-term homeostasis of the prostate. The aim of this study was to verify if exposure to BPA and 17-β estradiol during the perinatal window of susceptibility disrupts the inflammatory pattern of adult female prostate. Pregnant Mongolian gerbils (*Meriones unguiculatus*) were divided in three groups (n=5): control group (C); 17-β estradiol group (E2) - treated with 35 μg/kg in 0.1 ml corn oil (3 times/week); and BPA group - treated with 50 μg/kg of BPA in 0.1 ml corn oil (daily). The treatments were administered to the dams, beginning on the 8th gestational day until the last day of lactation, totaling 39 days of treatment. After weaning, one female was randomly chosen from each offspring, maintained without any other treatment and euthanized on the 180th postnatal day. Prostate was collected, fixed in paraformaldehyde 4% for 24 h, and processed. Immunohistochemical analysis for identification of inflammatory markers was performed. The incidence of positive cells per area was calculated (cells/mm²) and the results were expressed as means ± standard error. Despite no statistical differences were observed among the treated and control groups for Cox2 (C: 33.55±10.09; E2: 56.53±12.70; BPA: 91.53±29.88) and for TNFα (C: 5.47±0.53; E2: 41.47±21.03; BPA: 44.11±14.41) positive cells, the macrophage marker F4/80 was more expressed in the BPA (59.46±21.92) group in comparison to the C (C: 4.91±1.37) group. Similar results were observed for phospho-stat-3, where differences between BPA (33.10±6.77) and C (5.89±1.22) groups were found as well. These results show that perinatal exposure to the xenoestrogen BPA is able to cause long-term impacts in the inflammatory profile of the female prostate. Alterations in the inflammatory aspects of secretory glands, especially in the stromal compartments may prompt a tumorigenic microenvironment. Thus, our results suggest that the mechanisms involved in long-term malignant alterations caused by BPA perinatal exposure may be related to the presence of inflammation. Funding: this study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES) - Finance Code 001.

Hyperplasic and inflammatory repercussions on adult mammary gland after perinatal exposure to bisphenol A and 17-β estradiol

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Mammary gland (MG) is one of the most plastic organs in the female reproductive system and is highly responsive to endocrine mechanisms. Thus, endocrine disrupters, such as bisphenol A (BPA) and exogenous estrogens, negatively affect MG tissue. This study aimed to verify if proliferative and
Inflammatory repercussions are present in the MG of adult females after perinatal exposure to BPA and 17-β estradiol. Pregnant gerbils were divided in three groups (n=5): (IC) group - not treated; (E2) group - treated with 35 μg/kg of 17-β estradiol in 0.1 ml corn oil (3 times/week); and (BPA) group - treated with 50 μg/kg of BPA in 0.1 ml corn oil (daily). The mothers were exposed by gavage from eighth pregnancy day until the end of lactation (39 days). One female from each offspring was randomly chosen and euthanized at 180th day of age. MG was collected, fixed in paraformaldehyde 4% for 24 h, and processed for morphological and immunohistochemical analysis of inflammatory markers and myoepithelial cells. The incidence of intraductal hyperplasia was estimated by the percentage of epithelial structures with more than 3 layers of epithelial cells per total and the inflammatory markers was calculated by the number of positive cells per area. Data was checked for normality in the Kolmogorov-Smirnov test; parametric data were analyzed using one-way ANOVA followed by Tukey’s test. Lobuloalveolar hyperplasia was absent among IC animals and the incidence in E2 and BPA groups was 48.8% and 44.4%, respectively; in hyperplasic samples, myoepithelial cell layer was discontinuous and visibly thinner than in normal epithelium. The frequency of intraductal hyperplasia among in morphologically normal glands was respectively 4.6 ± 1.7, 21.5 ± 4.9 and 23.8 ± 5.7 for IC, E2 and BPA groups. However, there were no statistical differences among groups for the following inflammatory markers in the IC, E2 and BPA groups, respectively: TNFα (2.7±0.8; 2.8±1.1; 4.1±0.87); Cox-2 (3.0±1.0; 2.7±1.0; 2.8±0.4); F4/80 (1.1±0.2; 1.3±0.2; 1.640±0.6); and Phospho-STAT-3 (28.5±2.2; 33.5±15.6; 35.0±13.0). All markers showed cytoplasmic staining of immune cells in the stroma and Phospho-STAT-3 also showed a nuclear pattern of expression in epithelial cells. These results show that perinatal exposure to estrogeic compounds is capable of causing long-term proliferative lesions, but it does not impact the inflammatory response of MG. Future analysis will include Western blotting assay to quantify total amount of these proteins aiming to detect slight differences among the treated groups. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES) - Finance Code 001, and by the National Council for Scientific and Technological Development (Process number: 132059/2020-7).

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Impacts of beta-caryophyllene in the benign prostatic hyperplasia of adult Mongolian gerbils (Meriones unguiculatus).

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The prostate is very susceptible to changes in androgen levels, which can play an important role in the development of Benign Prostatic Hyperplasia (BPH). Many natural compounds have beneficial properties to the organisms and can be an important therapeutic strategy in the treatment of diseases. β-Caryophyllene (BCP) is a phytocannabinoid present in several plant species and has shown beneficial effects in different organs. Thus, the present study aims to evaluate the effects of exposure to BCP on the morphophysiology of the ventral prostate of adult gerbils (Meriones unguiculatus) supplemented by testosterone. Animals were distributed into the groups (n=7): Intact control (C); β-Caryophyllene (BCP): animals received for 30 consecutive days, via gavage, the β-Caryophyllene (50 mg/kg/day); Testosterone and β-Caryophyllene (TBCP): animals received, on alternate days and for one month, subcutaneous injections of Testosterone Cypionate (3 mg/Kg) and then, via gavage, for 30 consecutive days, the BCP (50 mg/kg/day); Immediate Testosterone (TI) and Late Testosterone (TT): animals received subcutaneous injections of Testosterone Cypionate (3 mg/Kg), on alternate days, for one month and...
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immediately euthanized after supplementation and 30 days after supplementation, respectively. Morphological, biometric, immunohistochemical, and serological analyzes were performed. Thus, it was possible to verify the presence of proliferative disorders and inflammatory foci in the epithelium and prostatic stroma of animals supplemented by testosterone and cellular rearrangement and tissue remodeling in the prostate of groups exposed to BCP. Was observed the reduction in the frequency of positive PHH3 and COX-2 markings in the prostatic epithelium of the TBCP group compared to the TT. The decrease in positive cells for macrophages F4/80 and CD163 marker was also observed in the prostatic stroma of the TBCP group in comparison to TT. Thus, the results suggest that β-Caryophyllene had beneficial effects on BPH, reducing the proliferation and frequency of some inflammatory cells. Therefore, we conclude that BCP influenced the tissue remodeling process and that the use of this phytocannabinoid can have positive results in the handling of BPH. CEUA protocol: No.173/2017. Financial support: CAPES, CNPq (Grant number 432360/2018-2).

Cell Growth, Senescence and Death in Aging

P667

HDAC6 mediates H₂S enhanced senescent wound healing
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Objective: Gasotransmitter hydrogen sulfide enhances senescent wound healing, the epigenetic regulatory mechanism is not understood. Methods: Endothelial cells and fibroblasts are two major types of cells that participate in wound healing. Primary human umbilical vein endothelial cells (HUVEC) and human dermal fibroblasts (HDF) are repeatedly subcultured to induce replicative senescence. Expression of histone deacetylase 6 (HDAC6), the scratch wound healing migration of both HUVEC and HDF with or without sodium hydrosulfide (NaHS, the H₂S donor) were studied. Tubastatin A, the specific HDAC6 inhibitor was used to explore the possible role of HDAC6 in NaHS mediated cell migration. Results: HDAC6 expression increases in senescent HUVEC, while decreases in senescent HDF when compared with young cells respectively. The scratch wound healing migration of both senescent HUVEC and HDF are impaired. NaHS accelerates migration in both young and senescent HUVEC, while it is only effective considering HDF migration in young cells. Interestingly, application of tubastatin A eliminate the effect of NaHS in migration of both cell types. Conclusion: Migration behavior of endothelial cells plays a key role in angiogenesis and wound healing process. Here we report for the first time that exogenous H₂S accelerates both young and senescent endothelial cell migration, which depends on HDAC6. Dermal fibroblasts, which is also essential in wound healing, their behavior are under regulation of H₂S as well, especially for the young cells, and HDAC6 is indispensable in this process.

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Targeting cellular stress to improve wound healing with age
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**Background.** Impaired or delayed skin wound healing with age represents a major clinical problem. One of the likely factors associated with poor wound healing is the lingering senescent cells in the wound. Although acute cellular senescence is known to promote wound healing, the increased burden of senescent cell accumulation with age triggers a stress response that negatively impacts tissue repair. Since fibroblasts play a crucial role in wound healing, we developed a senescent fibroblast populated collagen matrix (FPCM) to study the role of cellular senescence on wound healing. The objective of the study is to test interventional strategies to mitigate the effect of senescence, thereby improving wound healing. Senescent cells have been demonstrated to be resistant to apoptosis secondary to increased expression of anti-apoptotic BCL-2 proteins. We hypothesized that BCL-2 inhibition, along with a mitogen stimulation or nicotinamide supplementation, would improve the wound healing endpoints in the senescent FPCM model. **Methods.** Senescence in the FPCM model was induced either by replicative stress (RS) or oxidative stress (OS). Characterization of senescence was performed with fibroblast morphology assessment, senescence-associated beta-galactosidase activity (SA-β-Gal), proliferation assays, migration assays, fibroblast differentiation assay, and expression of senescence, DNA damage, and oxidative stress markers. The wound healing potential of the young and senescent FPCM was analyzed by monitoring the number of days required to heal the scratch wounds made in the matrices. The effect of BCL-2 inhibitor (ABT-737) and/or FGF2 (fibroblast growth factor 2) treatment on wound healing was determined. **Results.** We observed a delay in the wound healing response associated with an increase in β-Gal activity, expression of senescence, and DNA damage markers, α-SMA positive myofibroblasts, and a decrease in Ki-67 positive proliferating cells in the senescent FPCM compared to young FPCM (*p<0.001). Treatment of the senescent FPCM with ABT-737 reduced the fraction of senescent cells from the matrices and accelerated wound healing rate (*p<0.01). A combined ABT-737 and FGF2 treatment further improved the wound healing efficacy in the senescent models (*p<0.007). Also, we observed a decline in NAD⁺ level with senescence (*p<0.02). Supplementation with NAD⁺ precursor, NAM (Nicotinamide) rescued the delayed healing response. (*p<0.004). **Conclusion.** Taken together, the accumulation of senescent cells with age was associated with decreased healing efficacy. The reduction of these senescent cells with soluble factors improved the healing outcome in our model.

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**Proteomic analysis of senescence-like morphology induced by DNA damage in NIH3T3 cells**

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Cellular senescence is a stage of irreversible cell cycle arrest induced by DNA damage due to oxidative stress, UV radiation, and toxic substance exposure. In this study, we developed a cellular senescence model by inducing DNA damage through the DNA alkylating agent mitomycin C (MMC). We then performed a global proteomic analysis using the MMC-treated cells to identify the proteins involved in inducing morphological changes during cellular senescence. After 24 h of MMC treatment, NIH3T3 cells cultured in the growth medium for seven days served as the senescent cell model. In MMC-treated cells, the cell proliferation rate was found to be significantly reduced, and p21 expression was further confirmed. Moreover, in MMC-treated cells, positive reactions of aging-related γH2AX and β-galactosidase were found to be markedly induced, and the enlargement of cell nuclei and morphological changes were observed. Next, we examined the protein expression profile of MMC-treated senescent
cells and identified hundreds of proteins through proteomic analysis. Of all the identified proteins, 25 (e.g. fibronectin, calnexin, gelsolin, and lysosome-associated membrane glycoprotein-2) were upregulated and 14 (e.g. filamin A, plectin, and fatty-acid synthase) were downregulated upon MMC treatment. These proteins are known to regulate F-actin rearrangements, thereby suggesting that they might be involved in regulating cell morphology during senescence.

**P670**

Igf signaling-related gene expression is reduced in the telencephalon of aged zebrafish with memory decline

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The population ratio of over 65 years old is increasing in the world, the increment of patients with age-related dementia is one of the most serious problems in society. It is necessary to develop methods for preventing and treating dementia, but they have not been fully established. The main symptom of dementia is cognitive impairment which occurs at an early stage and gets worse gradually. To explore a target for the treatment of memory loss and learning disability, we focused on Insulin-like growth factor 2 mRNA-binding protein 3 (IGF2BP3, IMP-3). IGF2BP3 binds to the 5'-UTR of IGF-2 mRNA and activates its translation, and IGF-2 has a positive effect on memory. It is known that igf2bp3 mRNA expression level is decreased with aging in mammalian and zebrafish brain, however, the causal relationship between this change and age-dependent dementia is poorly understood. In this study, we examined changes in IGF signaling-related gene expression among brain regions of aged zebrafish with memory loss. First, we investigated at what age zebrafish show the decline of memory and learning ability as compared to young zebrafish (5-month-old). We evaluated cognitive function of zebrafish using fear conditioning test for 3 days, and aged zebrafish (21 or 23-month-old) showed memory loss as judged by a reduction in avoidance behavior of unconditioned stimulus (electric shock) after presenting conditioned stimulus (green light) throughout all the trials. Next, we compared IGF signaling-related gene expression by brain regions between young and aged zebrafish with memory decline. As a result, igf2a expression level was reduced with aging in all the regions (telencephalon, optic tectum, cerebellum, and diencephalon). The expression level of igf2b was decreased in the aged telencephalon, whereas it was increased in other regions. Furthermore, igf2bp3 expression was greatly reduced in the aged telencephalon as compared to other regions. Taken together, these results suggested that cognitive function might be impaired with aging due to the decreased activation of IGF signaling pathway, probably through the decreased igf2a, igf2b, and igf2bp3 expression levels in the telencephalon.

**P671**

Exploiting a gain-of-function SKN-1 mutant to discover novel mechanisms of transcription factor control

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SKN-1 is an essential transcription factor in C.elegans. Originally identified as a transcription factor essential for the specification of the intestine during development, SKN-1 has been characterized as the C.elegans orthologue of NRF2 in mammals. One primary function of SKN-1 includes the transcriptional
activation of phase two detoxification genes during oxidative and xenobiotic stress, however it has also been implicated in lipid metabolism. All of these essential cellular processes are needed for the maintenance of normal lifespan and as such, SKN-1 is a master cytoprotective transcription factor. Recently, while screening for novel regulators of SKN-1, several gain-of-function (gf) alleles of *skn-1* were identified. While these mutants have increased stress resistance early in life, they also display shorter lifespans than wildtype animals. These early findings suggest a link between uncontrolled regulation of cytoprotective transcription factors and disease pathology. We seek to define the molecular and cellular mechanisms that govern the regulation of SKN-1 by exploiting a constitutively active mutant. We show via fluorescent microscopy that phenotypes caused by SKN-1gf are not the result of an intense soma-wide accumulation of SKN-1gf. Additionally we performed ChIP-seq to determine the difference in genome occupancy between wildtype SKN-1 and SKN-1gf. Lastly we show that target gene expression of SKN-1gf is diminished in mutants defective for neurotransmitter release, suggesting that phenotypes associated with SKN-1gf are controlled by signaling between two head sensory neurons and other somatic tissues where SKN-1gf is expressed.

**P672**

**Apoptotic Cascades in the Inner Ear and Their Relationship to a Mobile Ca²⁺ Buffer (Oncomodulin)**  
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Age-related hearing loss (ARHL), or presbycusis, is a common condition among aging populations. Despite its prevalence, the fundamental cellular and molecular mechanisms involved in ARHL are not well-understood. Tight regulation of intracellular Ca²⁺, which is largely controlled by Ca²⁺ buffers and transporters, is essential to maintaining adult hearing function. Oncomodulin (OCM) is the predominant Ca²⁺ buffer in outer hair cells (OHCs) of the cochlea. Targeted deletion of *Ocm* leads to premature hearing loss and OHC loss in mouse models (Tong et al., 2016). Regardless of genetic background, OCM loss reduces hearing lifespan by greater than 50%. Caspase-dependent and caspase-independent apoptotic pathways have been implicated in ARHL. The literature suggests that ROS concentration increases throughout aging, leading to mitochondrial damage and subsequent release of pro- apoptotic factors. We hypothesize that OCM protects OHCs against cellular stress and apoptosis to delay ARHL. To address this hypothesis, we employed OHC-dependent hearing tests (DPOAE), genetically targeted fluorescent calcium sensors, western blot and immunocytochemistry to compare young and old *Ocm* WT and KO mice. We examined standard apoptotic markers for caspase-dependent and caspase-independent apoptosis - cleaved caspase -9 & -3 and EndoG, respectively. TOM20 was used to visualize the distribution and morphology of the mitochondria. Our results show an increase in mitochondrial cell stress markers in aged mice, which correlates to changes in OCM subcellular localization and expression. We observed a distinct re-localization of OCM from the cytoplasm to the nucleus of the OHC in old animals. These studies demonstrate that OCM plays a critical role as a Ca²⁺ regulator that is essential for hearing health by preventing OHC apoptosis associated with ARHL.

**P673**

**Loss of heterochromatin and retrotransposon silencing constitute an early phase in oocyte aging**  
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Mammalian oocyte quality reduces fast with female age. A well-studied aspect of this deterioration is an age-associated rise in oocyte aneuploidy. We show that prior to the occurrence of significant aneuploidy (at the age of 9 months in mouse females), epigenetic changes occur and impact oocyte quality and maturation ability. At this age- we observe a reduction in the staining for heterochromatin marks in mouse oocytes. This decrease is apparent in both constitutive heterochromatin and facultative heterochromatin marks, but is absent in active euchromatic marks which remain constant. We also show that a decrease of heterochromatin marks with age is apparent in human oocytes from IVF treatments. Heterochromatin loss with age is associated with an elevation in retrotransposon RNA transcription and processing, as detect in small RNA seq. Using IF staining, we detect an elevation in retrotransposon protein expression, an elevation in dsRNA foci and Dicer foci in the oocyte cytoplasm, occurrence of DNA damage. Aged oocyte IVM resulted in oocyte maturation defects. Artificial inhibition of the heterochromatin machinery in young oocytes causes an elevation in oocyte maturation defects. Inhibiting the retrotransposon reverse-transcriptase through Azidothymidine (AZT) treatment in older oocytes partially rescues their maturation defects and lowers their retrotransposon processing, but does not impact their heterochromatin loss, implying that the epigenetic defect acts upstream of retrotransposon activation at this stage in the aging process. Our working model is therefore that due to external damage and erosion with time, modified histones are lost from chromatin and replaced by non-modified nucleosomes. Silencing of wide genomic domains is eventually lost, and retrotransposon RNA is transcribed. This in turn causes an elevation in DNA damage, and a decrease in oocyte maturation.

Centrosome Assembly and Functions 2

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**Regulated redistribution of centrosomal protein by centromere attachment and polo kinase controls nuclear envelope breakdown in fission yeast**

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Proper mitotic progression in most eukaryotes requires nuclear envelope breakdown (NEBD) to allow the centrosome to access the chromosomes. In *S. pombe*, this is recapitulated by partial NEBD and insertion of the spindle pole body (SPB - yeast centrosome) to build the mitotic spindle. Linkage of the SPB to the centromere is vital to this process, but why that linkage is important is not well understood. Utilizing high-resolution Structured Illumination Microscopy (SIM), we show that the conserved SUN-protein Sad1 redistributes during mitosis to form a ring-like structure around the SPBs. Loss of either centromeric-SPB tethering proteins and/or loss of linkage between the centromere and the SPB leads to a loss of Sad1 SPB ring formation, showing that the vital role for this linkage is to ensure Sad1 redistribution during mitosis. We show that Sad1 redistribution precedes accumulation of other SPB components into a mitotic ring complex, which is necessary for NEBD and spindle formation. Interestingly, Polo Kinase is not necessary for Sad1 redistribution, but its function is vital redistribution of other SPB components into the mitotic ring complex and for complete NEBD. Taken together, our
data show that centromere linkage at the NE region near the SPB results in Sad1 reorganization, setting up a downstream set of events leading to NEBD and spindle formation through a novel mitotic ring complex regulated by Polo kinase.

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**PLK1- and PLK4-mediated asymmetric mitotic centrosome size and positioning in the early zebrafish embryo.**

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Factors that regulate mitotic spindle positioning remain unclear within the confines of extremely large embryonic cells, such as the early divisions of the vertebrate embryo, *Danio rerio* (zebrafish). We find that the mitotic centrosome, a structure that assembles the mitotic spindle, is notably large in the zebrafish embryo (246.44±11.93μm² in a 126.86±0.35μm diameter cell) compared to a *C. elegans* embryo (5.78±0.18μm² in a 55.83±1.04μm diameter cell). During embryonic cell divisions, cell size changes rapidly in both *C. elegans* and zebrafish, where mitotic centrosome area scales more closely with changes in cell size compared to changes in spindle length. Embryonic zebrafish spindles contain asymmetrically sized mitotic centrosomes (2.14±0.13-fold difference between the two), with the larger mitotic centrosome placed towards the embryo center in a Polo-Like Kinase (PLK) 1 and PLK4 dependent manner. We propose a model in which uniquely large zebrafish embryonic centrosomes direct spindle placement within disproportionately large cells.

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**Centrosome-Nucleated Microtubules Act in Parallel to Plk1 to Trigger Mitotic Entry**

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Entry into mitosis requires complex orchestration of multiple processes, two of which are activation of the Cyclin-dependent kinase 1 (Cdk1) and disassembly of the nuclear envelope. However, the critical initiating event in mitotic entry and the relationship between Cdk1 activation and nuclear envelope breakdown remain unclear. The Polo-like kinase Plk1 and centrosomes have both been proposed to play an important role during the transition into mitosis, yet whether Plk1 activity is essential for mitotic entry in human cells remains controversial, with conflicting results reported in different studies. To address the contribution of Plk1 and centrosomes to mitotic entry, we employed a live-cell imaging assay to measure the duration of G2, defined as the interval between the end of S-phase and nuclear envelope breakdown. Using two structurally distinct inhibitors (GSK461364 and BI2536), we found that stringent Plk1 inhibition delays but does not block mitotic entry. Centrosome removal, using an inhibitor of the Plk4 kinase that controls centrosome duplication, has no effect on G2 duration. Strikingly, a potent block to mitotic entry was observed when Plk1 was inhibited in centrosome-less cells. Disruption
of the microtubule cytoskeleton, like centrosome loss, also synergized with Plk1 inhibition in preventing mitotic entry and nuclear cyclin B1 accumulation. We hypothesized that Plk1 activity and centrosome-nucleated microtubules trigger mitotic entry by altering nuclear permeability. Consistent with this, our preliminary data shows that Plk1 and the nuclear pore-recruited pool of the microtubule motor dynein act in parallel to trigger mitotic entry. These results suggest that a Plk1-induced change in nuclear permeability is a key initiating event in mitotic entry. Although normally triggered by Plk1, this process can also be executed by centrosome-nucleated microtubules when Plk1 is inhibited. We are currently investigating what this event precisely is, and how it controls the activation of mitosis-triggering factors.

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Wait for me: Acentriolar cells require a functioning spindle assembly checkpoint to achieve bipolar spindle formation
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Centrioles are required to form the centrosome, a major microtubule organizing center (MTOC) during mitosis in animal cells. Mammalian cells manipulated to lack centrioles are still able to build bipolar spindles using acentriolar MTOCs (aMTOCs) and usually undergo bipolar divisions to produce two daughters. However, this acentriolar mitosis takes longer to complete, suggesting that it is not completely normal. We investigated how centriole loss elongates mitosis and whether the increased duration of mitosis is necessary for acentriolar cells to create bipolar spindles. We used two methods to eliminate centrioles in mammalian cells: inhibition of Polo-like kinase 4 (PLK4) or deletion of the gene for cartwheel component SASS6, both of which are individually necessary for centriole duplication. By observing mitosis in live acentriolar cells, we found that mitosis is not uniformly elongated, but rather early mitotic stages are elongated while later ones remain unchanged. Inhibition of the spindle assembly checkpoint (SAC) largely alleviated the mitotic elongation in acentriolar cells. However, acentriolar cells could not form bipolar spindles without a functioning SAC and failed to undergo either nuclear division or cytokinesis in the absence of SAC activity. In contrast, cells with centrosomes largely underwent bipolar nuclear division and cytokinesis even without SAC activity. We confirmed via live imaging of microtubules during mitosis that acentriolar cells achieve bipolarity of their aMTOCs much later than control cells with centrosome MTOCs. We were also able to recapitulate the elongated mitosis phenotype of acentriolar cells with a compromised SAC in cells with centrioles with impaired bipolar spindle formation. In conclusion, while cells with centrosomes typically achieve bipolarity of the MTOCs before the SAC acts, acentriolar cells do not have bipolar aMTOCs before the onset of SAC activity. Thus, the SAC acts as a necessary brake during acentriolar divisions that allows adequate time to establish bipolarity of aMTOCs, without which cell division cannot occur.

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‘Centrosome Reduction’, a process of meiotic centrosome disassembly requires Pericentrin degradation for male fertility
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‘Centrosome Reduction’ (CR) is an essential centriole remodeling process during human spermiogenesis. CR involves the removal of pericentriolar material (PCM) from the basal bodies prior to sperm formation, but the mechanism and biological significance of CR is unknown. Here, we use Drosophila model system to study CR, as this process appears to be highly conserved. We used quantitative fluorescence microscopy to carefully characterize a range of centrosome proteins, in particular, a group of PCM regulators known as the Bridge proteins (Pericentrin, Asterless, Spd2 and Sas4). We found that these bridge proteins are sequentially removed after PCM reduction in early spermatid development. We focused our study on Pericentrin given its known functions in sperm motility and male fertility. To characterize the mechanism of Pericentrin reduction, we performed a structure-function study of Pericentrin in developing spermatids. We found that the N-terminal region of Pericentrin harbors several reduction signals required for its removal from the basal bodies. Furthermore, we show that CR of Pericentrin is essential to prevent PCM tethering on basal bodies and for male fertility. Biochemical characterization revealed that these reduction signals of Pericentrin are canonical protein degradation signals. We therefore hypothesized that Pericentrin degradation is a part of CR mechanisms. To identify the precise degradation mechanisms, we performed a candidate RNAi screen comprised of APC, SCF and other degradation related proteasome components. We found Rad6 and its related E3 ligases to be required for Pericentrin degradation in male germ cells. In this study, we have shown a compelling evidence for Pericentrin degradation that facilitate CR in male meiosis, while our previous study by Galletta,2020 identified the importance of transcriptional control of Pericentrin for spatial restriction of PCM at the centrosomes. Collectively, our studies reveal Pericentrin homeostasis as a regulatory mechanism that controls centrosome maturation and the conserved CR pathway.

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orb2-dependent microcephaly reveals a novel role for RNA-binding proteins in centrosome regulation.

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During neurodevelopment, neural stem cells (NSCs) undergo repeated rounds of asymmetric cell division to maintain a population of self-renewing stem cells and generate the differentiating cells required for neurogenesis. In human and Drosophila NSCs, asymmetric centrosome activity ensures error-free mitosis along a defined polarity axis. Accordingly, loss of centrosome asymmetry is associated with errant microtubule organization and defective cell divisions. In humans, aberrant centrosome function is associated with the neurodevelopmental condition microcephaly, characterized by an abnormally small brain and head circumference. Centrosome asymmetries are generated through the asymmetric enrichment of the various proteins that comprise the pericentriolar material (PCM), which dictates the microtubule-organizing activity of the centrosome. The mechanism underlying regulation of centrosome activity is poorly understood. Intriguingly, the enrichment of specific mRNAs to the centrosome suggests that RNA localization coupled with local translation by RNA-binding proteins (RBPs) may contribute to centrosome regulation. We predicted that an RBP may bind the cognate RNAs of PCM proteins to regulate gene localization and expression. Through 3’ untranslated region (UTR) analysis of RNAs that localize to the centrosome, we identified the cytoplasmic polyadenylation element binding (CPEB) protein, Orb2, as a potential regulator of centrosome activity. CPEB proteins are implicated in mRNA localization and regulation of translation. Consequently, we examined a requirement for Orb2 in regulating NSC centrosome activities and overall neurodevelopment. Our preliminary data reveal that
**orb2** is a novel microcephaly gene. We found that **orb2** mutant brains have a volume of >2 standard deviations below the control mean. Additionally, **orb2** mutant NSCs show centrosome-associated phenotypes, including loss of centrosome asymmetry, centrosome amplification, and defective spindle morphology and orientation. Additionally, **orb2** mutant brains have a reduced number of NSCs compared to control brains, suggesting the observed microcephaly is a result of failed asymmetric cell division. These data are consistent with a novel role for Orb2, an RBP, in centrosome regulation.

**P680**

**The post-transcriptional regulations of centrosomal plp mRNA in Drosophila**

**J. Fang**, D. Lerit; Emory University School of Medicine, Atlanta, GA.

Centrosomes, functioning as microtubule organizing centers, are composed of a proteinaceous matrix of pericentriolar material (PCM) that surrounds a pair of centrioles. *Drosophila* Pericentrin (Pcnt)-like protein (PLP) is a key component of the centrosome that serves as a scaffold for PCM assembly. The disruption of plp in *Drosophila* results in embryonic lethality, while the deregulation of Pcnt in humans is associated with MOPD II and Trisomy 21. We recently found plp mRNA localizes to *Drosophila* embryonic centrosomes. While RNA is known to associate with centrosomes in diverse cell types, the elements required for plp mRNA localization to centrosomes remains completely unknown. Additionally, how plp translation is regulated to accommodate rapid cell divisions during early embryogenesis is unclear. RNA localization coupled with translational control is a conserved mechanism that functions in diverse cellular processes. Control of mRNA localization and translation is mediated by RNA-binding proteins (RBPs). We find PLP protein expression is specifically promoted by an RNA-binding protein, Orb, during embryogenesis; moreover, plp mRNA interacts with Orb. Importantly, we find overexpression of full-length PLP can rescue cell division defects and embryonic lethality caused by orb depletion. We aim to uncover the mechanisms underlying embryonic plp mRNA localization and function and how Orb regulates plp translation.

**P681**

**Pericentriolar matrix biophysical integrity relies on cenexin and Polo-Like Kinase (PLK) 1**

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Polo-Like Kinase (PLK) 1 activity is associated with maintaining the functional material properties of the centrosome’s pericentriolar matrix (PCM). However, what governs the spatial and temporal regulation of PLK1 at the centrosome remains enigmatic due to a multiplicity of interactions and substrates. In this study, we examine a PLK1-binding protein, cenexin, that localizes to a sub-centrosomal domain, the mother centriole appendages, where we identified that cenexin-dependent PLK1-mediated signaling events regulate centrosome architecture and function. Specifically, cells lacking cenexin either by shRNAs or a cenexin-null CrispR cell line display elevated amounts of active p-PLK1(T210) at mitotic centrosomes, fragmented PCM, a delay in microtubule-re-nucleation, and mitotic progression defects. The PCM fragments observed in cenexin-depleted cells were more fluid such that PCM fragments in cenexin-depleted cells can readily fuse with one another, and a fluorescent PCM marker, RFP-PACT, had a significant increase in its exchange rate at the PCM in cenexin-depleted cells when compared to
control. These phenotypes were rescued in cenexin-depleted cells with acute inhibition of PLK1, suggesting that these phenotypes are due to the elevated activity of PLK1 in cenexin-depleted cells. One downstream consequence of PLK1 inhibition and cenexin depletion are defects in bipolar spindle formation. We propose a model where cenexin anchors and constrains PLK1 to act on the correct substrates at the correct times.

P682

**Molecular and mechanical organization of cleavage pattern geometry in frog eggs**

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We seek to understand the mechanical basis of sub-cellular organization using eggs of *Xenopus laevis* as a model system. Cleavage geometry reports on organizing principles, but underlying molecular/physical mechanisms, and emergent mechanics, are poorly understood. We are focusing on two classic questions: how do furrows bisect the long axis of the cell and how are successive cleavage planes oriented at right angles? Furrows are positioned by pairs of egg-spanning microtubule asters that grow out from spindle poles at the end of mitosis. Centrioles at aster centers disjoin and move apart after anaphase. By the start of cleavage, each aster contains two centrosomes approximately 80 microns apart whose positions will determine the orientation of the subsequent mitotic spindle and furrow. This positioning of centrosome pairs inside asters determines the location of the 2<sup>nd</sup> cleavage plane before the first furrow starts to ingress. To understand how centrosomes separate and orient inside interphase asters we are imaging centrosome movement in normal vs. compressed eggs, perturbing potential force generators in an egg extract system and building mechanical models. We hypothesize that centrosomes are moved apart by local pushing forces that are blind to cell shape. In contrast, the orientation of the axis between them is controlled by global pulling forces exerted by organelle-bound dynein on the aster surface whose effects are sensitive to egg shape and anisotropies in aster mechanics.

**Channels and Transporters**

P683

**Receptor-like role for PQLC2 amino acid transporter in the lysosomal sensing of cationic amino acids**

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PQLC2, a lysosomal cationic amino acid transporter, also serves as a sensor that responds to scarcity of its substrates by recruiting a protein complex comprised of C9orf72, SMCR8 and WDR41 to the surface of lysosomes. This protein complex controls multiple aspects of lysosome function. Although it is known that this response to changes in cationic amino acid availability depends on an interaction between PQLC2 and WDR41, the underlying mechanism for the regulated interaction is not known. In this study, we establish that the WDR41-PQLC2 interaction is mediated by a short peptide motif in a flexible loop that extends from the WDR41 β-propeller and inserts into a cavity presented by the inward-facing
conformation of PQLC2. This data supports a transceptor model wherein conformational changes in PQLC2 related to substrate transport regulate the availability of the WDR41 binding site on PQLC2 and mediate recruitment of the WDR41-SMCR8-C9orf72 complex to the surface of lysosomes.

P684

**Characterization of metabolic gene expression in the RAW 264.7 monocyte cell line**

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Cellular metabolism is important because it consists of all the biochemical reactions occurring within cells and thus drives cellular processes and activity. During an immune response, white blood cells known as monocytes become activated, and it is hypothesized that their metabolic reactions will become more active to support the newly activated cellular processes. Substrates for metabolic reactions are delivered to cells using metabolic transporters. Therefore, the current study examined transporter proteins on monocytes in normal and reactive conditions. It was hypothesized that the expression of monocarboxylate transporter 1 (MCT1) and glucose transporter 1 (GLUT1) would increase during an immune response. The RAW 264.7 cell line was treated with lipopolysaccharide (LPS, 1 μg/mL) from Gram negative bacteria or saline for 24 hours. The cells were fixed and subjected to immunocytochemistry using antibodies specific for MCT1 and GLUT1. The cells were visualized using a laser scanning confocal microscope using the same excitation and emission conditions for all treatments and the relative fluorescence was measured using the accompanying software. It was determined that the expression of MCT1 is significantly greater (1.75×) than that of GLUT1 in RAW 264.7 cells in saline control conditions. Transporter expression did not completely reverse in response to LPS, however significant differences in expression were observed for both GLUT1 and MCT1. The expression of GLUT1 was significantly increased in response to LPS, when compared to the saline control (PBS/LPS = 1.05× increase). Concomitantly, the expression of MCT1 was significantly decreased in response to LPS, when compared to the saline control (PBS/LPS = 0.92× decrease). The results suggest that a metabolic switch does occur in monocytes during an immune response, from that of general cell maintenance with greater dependence on monocarboxylates like lactate, pyruvate, and ketone bodies, to a heighten response with an increased dependence on glucose.

P685

**Novel insights into the transport mechanism of the zinc transporter ZupT from Escherichia coli and implications on human ZIP transporters**

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Zinc (Zn$^{2+}$) and iron (Fe$^{2+}$) are essential nutrients. Their uptake and distribution into cells are critical for life, and are carried out in part by zinc regulated, iron regulated like proteins (ZIP) transporters. ZIP transporters are responsible for increasing the cytoplasmic level of a broad range of divalent cations notably Zn$^{2+}$, Fe$^{2+}$, and Mn$^{2+}$, and the transport of the toxic metal Cd$^{2+}$. This important protein family can be found in all phyla of life and is essential for human health. The human ZIP transporters can be subdivided into four subgroups; one of these, the gufA subfamily, is the most ancient member of the ZIP family. The gufA subfamily is represented in humans by ZIP11 and in *Escherichia coli* by ZupT. ZupT has been shown to transport Zn$^{2+}$, Fe$^{2+}$, and Cd$^{2+}$, but the mechanism of transport is not well understood.
We investigated the transport activity of ZupT for Zn\textsuperscript{2+}, Fe\textsuperscript{2+}, and Cd\textsuperscript{2+} using \textit{in vivo} and \textit{in vitro} activity assays. We also performed metal binding analysis coupled with site-directed mutagenesis to determine which residues are important in metal recruitment, binding and release. Our findings identified critical amino acids in metal transport that are largely conserved in the ZIP family. This study represents the first known instance of a comprehensive \textit{in vitro} metal binding analysis of a ZIP transporter, and insights into how Zn\textsuperscript{2+} transport in ZIP proteins may be regulated. These findings reveal essential aspects of ZIP transporters at their most basic level, which are likely shared among all members. Studying this transporter will help elucidate the interaction between Zn\textsuperscript{2+} and Fe\textsuperscript{2+} in maintaining total metal cellular homeostasis.

P686

\textbf{A genome-wide CRISPR/Cas9 screen identifies novel regulators of GSDMD pore formation in engineered macrophages}

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Cleavage of gasdermin D (GSDMD) by inflammatory caspases results in pore formation at the plasma membrane. GSDMD pores are recognized to mediate pyroptotic lysis of the cell or direct translocation of IL-1 family cytokines from the cytosol into the extracellular space depending on the quantity of pores or duration of plasma membrane occupancy. Hyperactive cells display evidence of fewer GSDMD pores compared to their pyroptotic counterparts, and recent work suggests that ESCRT-dependent membrane repair pathways oppose the cell fate towards pyroptosis through removal of GSDMD pores from the plasma membrane. To identify regulators of GSDMD pore formation at the plasma membrane, we utilized a genome-wide CRISPR/Cas9 screening platform in immortalized bone marrow derived macrophages (iBMDMs). This screen uncovered novel regulators of GSDMD pore formation in the plasma membrane.

P687

\textbf{Localization of YFP-Tagged ABC Transporters in Tetrahymena thermophila}

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The \textbf{ATP-Binding Cassette} (ABC) transporters are a large family of ATP-driven pumps found in all cells. ABC transporters are diverse and transport a wide range of substrates, from ions to proteins to environmental toxins, across cellular membranes. Eukaryotic ABC transporters typically consist of two types of domains, a nucleotide-binding domain (NBD) and a transmembrane domain (TMD). The freshwater ciliate \textit{Tetrahymena thermophila} has 165 ABC transporters, more than any other organism studied to date, and little is known about the functions of most of these transporters. ABC transporters are classified into one of eight families, ABCA through ABCH. We chose representatives of three of these classes from \textit{Tetrahymena}, ABCB12, ABCF1 and ABCG34, for further study. Both ABCB12 and ABCG34 have a single NBD and a single TMD, and ABCF1 has two NBDs and lacks a TMD. Based on homology to transporters in yeast, humans and plants, we hypothesized that ABCB12, ABCF1 and ABCG34 localize to the mitochondria, cytosol and plasma membrane, respectively. We overexpressed YFP-tagged genes for
these proteins and confirmed these localizations. In addition to the plasma membrane, ABCG34 targeted a variety of vacuolar membranes. To further study the function of these three transporters in *Tetrahymena*, we created plasmids to knockdown the expression of these genes. Our initial studies show that growth is not significantly affected in any of the knockdown strains, and future work will be aimed at determining more subtle phenotypes in the knockdown strains to better understand the function of these transporters.

P688

**Piezo mechanosensitive ion channel homologs regulate vacuolar morphology in plants**

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Perception of mechanical forces is a fundamental property of most, if not all cells. One way this is achieved is through the action of mechanosensitive (MS) ion channels, which open in response to lateral membrane tension. Piezo channels are mechanically gated, plasma membrane-embedded calcium/cation channels that play diverse and essential roles in numerous mechanobiological processes in animals. Piezo channel homologs are found in plants, but their role(s) in the green lineage are almost completely unknown. Plants and animals diverged approximately 1.5 billion years ago, independently evolved multicellularity, and have vastly different cellular mechanics. Plant cells have a cell wall and up to a thousand-fold higher osmotic pressure than animal cells - so how Piezo homologs might function in plant cells is an especially intriguing question. The moss *Physcomitrium patens* has two Piezo homologs, PpPiezo1 and PpPiezo2, likely products of recent gene duplication. Unlike animal Piezo channels, both PpPiezo1 and PpPiezo2 localized to the vacuolar membrane. Vacuoles are large aqueous organelles that store ions and metabolites, maintain cellular osmotic potential and pH, and are part of the degradation pathway. We used CRISPR/Cas9 gene editing to generate loss-of-function (ΔPpPiezo1/2) and gain-of-function (PpPiezo2-R2508K and -R2508H) mutants. The tip-growing caulonema cells of ΔPpPiezo1/2 mutant moss lines had reduced growth rates and altered cellular morphology. Their vacuoles were larger and more expanded, unlike the tubular vacuoles of the wild type cells. When moss was exposed to multiple stress conditions, the vacuoles in WT caulonema cells also expanded, phenocopying ΔPpPiezo1/2 mutants. Conversely, the vacuoles of PpPiezo2-R2508K/H mutant caulonema cells were often highly laminated with complex internal membrane structures. Taken together, our data suggest that PpPiezo1 and PpPiezo2 regulate vacuolar morphology. As plant vacuoles are large stores of calcium, we tested the calcium responses in ΔPpPiezo1/2 mutants and found that these moss lines have a reduced calcium response to sudden hypoosmotic shock. Thus, plant Piezo homologs have diverged both in cellular localization and function from their animal counterparts and may have been co-opted to sense the mechanical status of the vacuolar membrane. Like animal Piezo channels, plant Piezos may still release calcium into the cytosol, just from vacuolar rather than extracellular stores. Perhaps this divergence reflects the higher freedom of movement of vacuolar membranes compared with the plasma membrane in plant cells, making them a preferred location to sense and respond to mechanical changes within the cell.
Cilia Signaling

Ankmy2 prevents Smoothened independent hyperactivation of hedgehog pathway via cilia regulated adenylyl cyclase signaling

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The mechanisms underlying subcellular targeting of cAMP-generating adenylyl cyclases and processes regulated by their compartmentalization are poorly understood. The primary cilium is a microtubule-based dynamic cellular appendage that mediates extracellular signaling and morphogenesis by hedgehog (Hh) ligands in vertebrates. An intricate balance between Gli transcriptional activators and repressors determines the transcriptional output of the Hh pathway. Binding of Hh to its receptor Patched triggers enrichment and activation of Smoothened—the pathway transducer—in cilia, generating Gli activator. In contrast, repression involves protein kinase A (PKA)-regulated limited proteolysis of full-length Gli2/3 proteins into Gli repressors, also requiring cilia. However, a poor understanding of key cilia-dependent regulators of cAMP/PKA signaling, redundancy between multiple ACs, and the lack of methods to study and perturb ciliary signaling, while maintaining intact cilia, have precluded from a clear understanding of repression of Hh signaling during neural tube development. We previously described that the cilia-localized orphan GPCR, Gpr161 functions as a repressor of Hh signaling via Gαs-coupled cAMP signaling and Gli3 repressor formation. Here, we report Ankmy2, an ankyrin-repeat and MYND-domain containing protein, as a major repressor of Hh pathway via AC targeting. Ankmy2 bound to multiple ACs, determining their maturation from core to complex glycosylated forms in the secretory pathway and eventual trafficking to cilia. Mice lacking Ankmy2 were mid-embryonic lethal by the 10-12-somite stage at embryonic day (E) 8.5, much before lethality of the Gpr161 knockout (ko) embryos at E10.5. Ankmy2 ko embryos had increased Hh signaling, completely open neural tube (craniorachischisis) and showed Hh-dependent ventral cell types ectopically specified at the expense of lateral and dorsal cell types (ventralization), comparable to loss of Patched. Ventralization in Ankmy2 ko, even of the most ventrally expressed floor plate markers that are considered to be strictly Hh-dependent, was completely independent of Smoothened. Instead, reduced levels of Gli2/3-repressors and early depletion of ACIII in neuroepithelial cilia, even before somitogenesis, implicated deficient Hh pathway repression. However, ventralization and open neural tube defects in Ankmy2 ko was dependent on cilia and Gli2 activation. Ciliary tips of Ankmy2 ko cells had Gli2 accumulation, even without Smoothened activation, which was suppressed by dibutryl-cAMP. These findings indicate that cilia-dependent AC signaling represses the Hh pathway by preventing default Gli2 activation and establishes a determinative role for pathway repression in morphogenetic patterning.

Ubiquitination Regulates Ciliary Localization of Smoothened

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Cilia monitor the extracellular environment through ciliary-localized receptors allowing the cell to coordinate its physiology with the environment and surrounding cells. Hedgehog signaling, the best studied ciliary pathway, plays fundamental roles during development. Abnormal hedgehog signaling causes many of the developmental defects due to ciliary dysfunction. The key components of the pathway are enriched in the cilium and their localization changes in response to pathway activation. In the basal state, patched-1 (Ptch1) accumulates in cilia and prevents smoothened (Smo) ciliary accumulation and activation. Upon binding of ligand, Ptch1 exits the cilium, Smo is derepressed and accumulates in the cilium, subsequently activating the downstream signaling. This leads to accumulation of Gli transcription factors at the ciliary tip before their modification and translocation to the nucleus where they modulate expression of target genes. While hedgehog signaling is widely studied, mechanisms controlling the ciliary levels of its components are poorly understood. Their movement is partly facilitated by intraflagellar transport (IFT) and perturbing IFT disrupts hedgehog signaling. IFT, which is critical for ciliary assembly and maintenance, involves motor driven transport of IFT particles consisting of ~30 proteins organized from IFT-A, IFT-B, and BBSome subcomplexes. The IFT particle provides binding sites for diverse ciliary cargoes. Ift25 and Ift27, subunits of IFT-B, are not required for ciliary assembly. Instead, they work with Lztfl1 and the BBSome to regulate hedgehog signaling and maintain proper levels of Smo and Ptch1 in cilia during signaling. Defects in intraflagellar transport components, including Ift27 and the BBSome, cause Smo to accumulate in cilia without pathway activation. We find that in the absence of ligand-induced pathway activation, Smo is ubiquitinated and removed from cilia, and this process is dependent on Ift27 and BBSome components. The activation of Hedgehog signaling decreases Smo ubiquitination and ciliary removal, resulting in its ciliary accumulation. Blocking ubiquitination of Smo by an E1 ligase inhibitor or by mutating two lysine residues in intracellular loop three causes Smo to aberrantly accumulate in cilia without pathway activation. Based on this data, we propose a model where Smo that enters the cilium at the basal state becomes ubiquitinated by an unknown E3ligase. Ubiquitinated Smo is recognized as cargo for retrograde IFT leading to its cilium exit and preventing Smo accumulation in cilia in the absence of pathway activation. Ptch1 may be regulated by a similar mechanism working after pathway activation.

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**Primary cilia sensitize insulin receptor-mediated negative feedback in pancreatic beta cells**

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Insulin receptors can localize to the primary cilia of pancreatic beta cells. Because primary cilia are known to sensitize or bias the signaling of cell surface receptors, we investigate how ciliary insulin receptors influence glucose-stimulated insulin secretion (GSIS) in beta cells. Contrary to the previous reports that insulin promotes insulin secretion by elevating cytosolic calcium, we find that a high insulin concentration inhibits calcium influx in a mouse insulinoma cell line (MIN6). Insulin receptors accumulate in the primary cilia of MIN6 cells in an activation-dependent manner. Interestingly, ciliated cells show attenuated cytosolic calcium elevation compared to cilium-free cells after glucose stimulation in the absence of exogenous insulin. The attenuation is abrogated by pretreating cells with an insulin receptor inhibitor, BMS536924. Furthermore, disruption of ciliary function by RNAi, targeting either IFT88 or BBS1, blocks both the ciliary enrichment of insulin receptors and the ciliary-dependent

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inhibition of calcium elevation. Thus, our results argue that primary cilia sensitize insulin receptor signaling and mediate negative feedback in GSIS in pancreatic beta cells.

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Characterization of ciliary targeting of rhodopsin-like GPCRs using a novel localization sequence in the SSTR3 C-terminus
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Primary cilia mediate critical sensory and developmental signals via the specialized localization of membrane proteins, including G-protein coupled receptors. It is unclear how this specialized localization is accomplished. We have discovered a 20 amino acid ciliary localization sequence (CLS) in the C-terminus of somatostatin receptor-3 (SSTR3). In IMCD3 cells, we found that removal of a segment of the C-terminus of SSTR3 results in reduced ciliary localization. Replacement of the C-terminus of Rhodopsin (RHO) with the SSTR3 CLS enhanced its ciliary localization, while addition of the SSTR3 CLS to CD8a-EGFP was sufficient to impart ciliary localization. Using structured illumination microscopy (SIM), we have found that EGFP-tagged RHO and CD8a localize to the ciliary pocket of hTERT-RPE1 cells, rather than in the ciliary membrane. Removal of the SSTR3 CLS also resulted in localization of SSTR3-DeltaCt-EGFP in the ciliary pocket. Replacement of the RHO C-terminus with the SSTR3 CLS resulted in localization to the ciliary membrane, and addition of the SSTR3 CLS to CD8a-EGFP resulted in a similar effect. TULP3 is implicated in GPCR localization to the cilium, including SSTR3, however the sequence that mediates TULP3-dependent SSTR3 localization is unknown. We generated a TULP3 knockout cell line, and found that ciliary localization of RHO- and CD8a- chimeras containing the SSTR3 CLS was greatly diminished in the TULP3 knockout, an effect that could be rescued by expression of TULP3. In conclusion, using a novel TULP3-dependent ciliary localization sequence, we have found that proteins with poor ciliary localization are localized to the ciliary pocket of hTERT-RPE1 cells, but may be redistributed to the cilium membrane with the addition of a CLS.

Cytokinesis: Other Model Organisms

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Cooperation between actin filament crosslinkers during cytokinesis
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The actin cytoskeleton is essential for a wide range of processes that require force generation and cell shape changes. Generation and efficient propagation of contractile force requires myosin II motors and non-motor crosslinkers: bipolar myosin II filaments slide actin filaments past one another at the expense of ATP, while crosslinkers maintain the connectivity of the F-actin network during filament sliding. Cytokinesis is the last step of cell division that physically partitions the mother cell into two daughter cells. This process relies on the assembly and constriction of a specialized F-actin network, the contractile ring. Continued constriction of the contractile ring pulls the plasma membrane inwards until the daughter cells are separated. The contractile ring is an F-actin circumferential array that includes non-muscle myosin II motors and F-actin crosslinkers. While it is well established that myosin is essential for cytokinesis in animals cells, the involvement of F-actin crosslinkers, which are in principle central
modulators of actomyosin contractility, remains poorly explored. We find that individual depletion of several F-actin crosslinkers does not preclude cytokinesis in the early *C. elegans* embryo. As several F-actin crosslinkers are expressed in the one-cell *C. elegans* embryo, we aim to understand how the crosslinker combination influences network contractility and force propagation during cytokinesis. To understand whether compensatory or synergistic effects between crosslinkers exist, we are using genetics to combine F-actin crosslinker mutants and assessing by live microscopy and quantitative analysis the impact of affecting more than one crosslinker at a time. Our findings unravel novel contributions of crosslinkers to cytokinesis and reveal that cooperation between crosslinkers with different mechanical properties is key for the proper functioning and contractility of the actomyosin network during cytokinesis.

P694

**The Rho-dependent Anillin-septin network facilitates contractile ring closure and disassembly during cytokinesis.**

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Animal cell cytokinesis proceeds through assembly and closure of an actomyosin contractile ring (CR) at the cell equator, which constricts to cleave the cell. However, despite nearly 50 years of research, the specific mechanisms of how the CR closes and disassembles as it constricts, remain elusive. Activation of the small GTPase Rho is known to drive the assembly of actomyosin. Moreover, Rho activation also controls the recruitment of the scaffold protein Anillin and septins to the CR, independently of actomyosin. However, the roles and regulation of the Rho-dependent control of Anillin at the CR are not understood. This study probes the mechanisms of contractile ring closure and specifically investigates the mechanism of septin recruitment to the CR by the scaffold protein Anillin, and in turn septin-driven removal of Anillin from the ring. Time-lapse imaging of dividing *Drosophila* S2 cells revealed that a point mutant of Anillin unable to bind to Rho-GTP (Anillin-RBD*), while still able to localize to the CR, failed to recruit septins to the contractile ring. Anillin-RBD* also did not support the formation of Rho, Anillin and septin-dependent cortical structures that form at the equatorial membrane upon LatrunculinA-induced depolymerization of actin. Closure of CRs dependent upon Anillin-RBD* was significantly slowed and did not exhibit the typical septin-dependent membrane extrusion and shedding that normally accompanies CR disassembly and maturation of the subsequent midbody ring. Furthermore, cells dependent upon Anillin-RBD* exclusively failed cytokinesis while harboring an aberrant internal midbody ring-like structure. Together, these data suggest that active Rho binding to Anillin is a prerequisite for septin binding and subsequent recruitment to the contractile ring. As the CR constricts, septins then drive the removal and shedding of Anillin and its associated membrane from the CR to facilitate its closure and disassembly. The data testify to the existence and highlight the importance of another Rho-dependent cytoskeletal sub-network: the Rho-Anillin-septin sub-network that, together with the actomyosin sub-network, makes up the contractile ring and facilitates its closure, disassembly and allows successful completion of cytokinesis.
Fundamental differences caused by cell size and fate promote unique cytokinesis kinetics in the two-cell *C. elegans* embryo

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Cells divide throughout embryogenesis and mitosis must be tightly regulated to ensure correct cell fate and prevent aneuploidy. The prevailing dogma is that spindle-dependent pathways regulate cytokinesis, based primarily on studies using cultured cells or pluripotent one-cell embryos. However, spindle-independent mechanisms also regulate cytokinesis, and their requirement may vary with cell fate, shape or size. We are using the two-cell *C. elegans* embryo, which has an anterior AB cell fated to be multiple tissues and a posterior P1 cell fated to be germline, to elucidate differences in the mechanisms regulating cytokinesis between cells with different fates. We uncovered different thresholds of actomyosin that support ring assembly in AB vs. P1 cells. Levels of cortically accumulated and midplane myosin are lower and differently organized in P1 cells, which have a longer assembly phase than AB cells where myosin accumulates in large patches as well as filaments. AB cells also ingress asymmetrically compared to P1 cells, which could occur due to differences in how the patches generate contractility and/or connect filaments. The patches are lost upon ect-2 (RhoAGEF) depletion, and AB cells that fully ingress have longer assembly phases and minimum thresholds that resemble control P1 cells. Cytokinesis is equalized upon depletion of polarity regulators (par-1, -3 or -6) supporting that their differences in assembly are cell-fate-dependent. To determine the contribution of size to the different kinetics of AB vs. P1 cells, we generated tetraploid embryos with properly fated, but larger, AB and P1 cells. The duration of ring assembly in tetraploid P1 cells was faster, similar to diploid AB cells, but surprisingly much slower in tetraploid AB cells, revealing cell-fate-dependent differences in the correlation between ring assembly and size. Our lab discovered a chromatin-sensing Ran pathway that functions redundantly with spindle pathways in cultured mammalian cells. Our model is that cortical proteins with nuclear localization signals are recruited equatorially to position the ring at a distance from chromatin where Ran-GTP levels are high. We found that depletion of ran-3 (RanGEF; RCC1) caused AB and P1 cells to have equally rapid assembly phases without altering their fate or dramatically altering their size. These kinetics were slowed by co-depletion of contractility regulators ect-2 or let-502 (Rho Kinase). Co-depletion of ani-1 (anillin, a contractile ring scaffold) suppressed cytokinesis phenotypes in AB but not P1 cells, supporting different pathway requirements in these cells. Our findings reveal cell-fate and -size dependent differences in mechanisms regulating cytokinesis and emphasize the need to study cytokinesis in different cell types.

Premature cleavage of the cytokinetic bridge disrupts lumen formation *invivo*

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Multicellular rosettes are transient epithelial structures that serve as intermediates during diverse organ formation. We identified that the final stage of mitosis termed abscission is a unique contributor to rosette formation in the zebrafish Kupffer’s Vesicle (KV). Following furrow ingression, animal cells stay interconnected by an intracellular bridge that contains a proteinaceous structure called the midbody.
The process by which this intracellular bridge is cleaved is termed abscission. We determined that percentage of midbodies at the apical surface significantly increases as KV develops from pre-rosette to rosette to lumen stages, demonstrating that the cytokinetic bridge constricts toward the apical lumen and suggesting a role for the placement of cytokinetic bridge in lumen formation (Rathbun et al., 2020). Using live cell confocal microscopy, we note that in a KV destined cell exiting mitosis, the two newly formed daughter cells utilize the cytokinetic bridge to orient themselves such that the cytokinetic bridge is placed where the lumen will form (Rathbun et al., 2020). At this time, the bridge is cleaved on one side of the midbody and then the other depositing the midbody into the lumen (Rathbun et al., 2020). Strikingly, premature severing of the cytokinetic bridge through the targeting of a single midbody at the site of lumen formation during apical clustering or the section next to the midbody of a resolved cytokinetic bridge using laser ablation results in severely diminished or failed lumen formation (Rathbun et al., 2020). We present a model in which KV mitotic cells strategically place their cytokinetic bridges at the rosette center to aid in lumen establishment.

P697

Decreasing cortical F-actin connectivity facilitates the constriction of contractile rings with breaks in the actomyosin structure

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Cytokinesis is the process that allows for cell division by cleaving the mother cell into two daughter cells. The process relies on the assembly and constriction of an actomyosin contractile ring at the equator of the mother cell. The contractile ring is integrated in the surrounding actomyosin cortex and therefore a global view of cytokinesis should involve the understanding of both the contractile ring and the surrounding cortex. In our previous work, we showed that cutting constricting rings open by laser microsurgery leads to local ring snapping followed by repair of the ring structure and continued constriction. Analysis of ring behavior after cutting indicated that overall cortical tension resists ring constriction, especially during the early phases of constriction (Silva et al., 2016). To gain further insight into how the surrounding cortex contributes to cytokinesis, we examined constricting rings in C. elegans 4-cell embryos partially depleted of the formin CYK-1, which nucleates the actin filaments in the contractile ring. CYK-1 penetrant depletion prevents the contractile ring from forming but its partial depletion allows for the formation of contractile rings that present decreased actin levels and slow constriction (Chan et al., 2019). Close inspection of CYK-1-depleted contractile rings revealed the presence of spontaneous breaks, which resemble the breaks induced by laser microsurgery. Breaks are observed throughout ring constriction but are more frequent early on, and multiple breaks can form simultaneously at any location around the ring circumference. Decreasing cortical tension by decreasing the connectivity of the actin filament network attenuates break formation in the contractile ring and allows for faster ring constriction. Our data support the idea that the surrounding cortex impacts contractile ring behavior and that a compromised ring made of substantially less actin filaments can constrict robustly when resistance from the surrounding cortex is decreased. Silva AM, Osório DS, Pereira AJ, Maiato H, Pinto IM, Rubinstein B, Gassmann R, Telley IA, Carvalho AX. Robust gap repair in the contractile ring ensures timely completion of cytokinesis. J Cell Biol. 2016 Dec 19;215(6):789-799. doi: 10.1083/jcb.201605080 Chan FY, Silva AM, Saramago J, Pereira-Sousa J, Brighton HE, Pereira M,

P698

The *C. elegans* zygote cytokinetic ring undergoes contractile oscillations

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The actomyosin contractile cytoskeleton is an active material that drives tissue and cell shape changes in animals and fungi. Irreversible cell shape changes such as cytokinetic furrowing during cell division are driven by actomyosin contractility, and regulated by the molecular switch RhoA. It is unknown how transient pulses of RhoA activation are translated into sustained contraction of large cytoskeletal assemblies such as the cytokinetic ring. To gain novel insights into cytokinetic ring dynamics, we imaged cytokinesis with unprecedented temporal resolution in the *C. elegans* zygote. The cytokinetic ring did not exhibit uniform contractile behavior but instead, furrowing underwent repeated cycles of acceleration and deceleration. While the entire ring circumference exhibited speed oscillations, at any given time, a singular site on the ring ingressed fastest and had the highest amplitude oscillations. The circumferential location of highest speed and amplitude travelled circumferentially over time. Depletion of conserved structural and regulatory cytokinetic ring components only subtly changed oscillation frequency, but dramatically affected amplitude. Non-muscle myosin II and its activator Rho-kinase drove oscillation amplitude and the non-motor crosslinker plastin/fimbin, as well as the formin family actin nucleator CYK-1 attenuated oscillation amplitude. These oscillations and traveling wave point to negative feedback loops in the cytokinetic actomyosin cytoskeleton. Thus, defining the molecular requirements for oscillation period and amplitude will provide insight into negative regulation in cytokinesis.

Cytoskeletal Signaling in Morphogenesis

P699

A hierarchy of protein patterns robustly decodes cell shape information

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Many cellular processes rely on precise positioning of proteins to ensure proper functioning. Such protein patterns are susceptible to cell-shape changes, raising the question of how these patterns can robustly regulate cellular tasks including cell division. Here, we elucidate a shape-adaptation mechanism that robustly controls spatiotemporal protein dynamics on the membrane despite cell-shape deformations. By combining experiments on starfish oocytes with biophysical theory, we show how cell-shape information contained in a cytosolic gradient can be decoded by a bistable regulator of Rho. In turn, this bistable front precisely controls a mechanochemical response by locally triggering excitable dynamics of Rho. We posit that such a shape-adaptation mechanism based on a hierarchy of protein patterns may constitute a general physical principle for cell-shape sensing and control.
P700

**Dynamics of altruistic fluid transport in egg development**

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Across species, oocytes develop within germline cysts alongside nurse-like sister cells. Prior to fertilization, the nurse cells donate their cytoplasm to the oocyte, which grows as its sister cells regress and die. Although critical for fertility, the biological and physical mechanisms underlying this transport process are poorly understood. Here, we combined live imaging, genetic perturbations, and flow-network theory to investigate the directional and complete cytoplasmic transport dynamics in 16-cell *Drosophila* germline cysts. We discovered that during ‘nurse cell (NC) dumping’, intercellular cytoplasmic transport dynamics is predicted by Young-Laplace’s law, consistent with NCs exhibiting a baseline surface tension, and independently of changes in myosin-II contractility. Long thought to drive transport through ‘squeezing’, increased actomyosin contractility is required only once NC volume is reduced by ~75%; myosin-II mediated contractions then appear as cell-scale surface waves that dynamically deform NC shape, cause membrane blebbing, and enable cytoplasmic transport to run to completion. Furthermore, we have identified a RhoGAP that is required for actomyosin waves and are investigating its spatiotemporal dynamics during NC dumping. Our work thus demonstrates how biological and physical mechanisms cooperate to promote a conserved act of cytoplasmic transport in early development.

P701

**Dunk interacts with anillin and regulates its cortical localization during *Drosophila* cellularization**

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Cleavage is a common step of early embryonic development, generating a monolayer of epithelial cells at the surface of the embryo called “blastoderm”. In *Drosophila*, this process is achieved by cellularization, a special form of cytokinesis that partition the peripherally localized syncytial nuclei into individual cells. Similar to typical animal cytokinesis, cellularization is initiated by recruiting non-muscle myosin II (“myosin”) to the cleavage furrows, which involves a cortical flow of myosin towards the leading edge of the newly formed furrows. We have previously identified a gene *dunk* that is required for cortical retention of myosin during the course of myosin flow, but the underlying mechanism is unclear. Through a genome-wide yeast two-hybrid screen, we identified anillin (Scraps in *Drosophila*), a conserved scaffolding protein involved in cytokinesis, as the primary binding partner of Dunk. Dunk binds to the highly conserved C-terminal domain of anillin, which also contains binding sites for several important regulators for anillin, including Rho1 and PI(4,5)P₂. Anillin has been previously shown to colocalize with myosin at the cellularization front, but it is unclear how early the two proteins colocalize to each other and whether anillin facilitates myosin recruitment to the nascent furrows. Using live imaging, we found that anillin colocalizes with myosin at the nascent furrows at the onset of cellularization. Embryos maternally mutant for *anillin* showed a cortical myosin loss phenotype during
early cellularization, closely resembling the dunk mutant phenotype. Several lines of evidence suggest that anillin and Dunk function in the same pathway during cellularization. First, in dunk mutant embryos, the localization of anillin at the cleavage furrows is severely disrupted. Second, there is a genetic interaction between anillin and dunk as revealed by embryos doubly heterozygous for the two genes. Finally, dunk and anillin mutants showed similar synthetic effects when combined with the loss of Bottleneck, an actin cross-linking protein controlling actomyosin organization during cellularization. Taken together, we propose that Dunk facilitates cortical myosin retention during early cellularization by interacting with anillin and regulating its cortical localization.

P702

**Multi-tissue patterning drives anterior morphogenesis of the C. elegans embryo**

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We characterized the cell patterns that arise during anterior morphogenesis in *C. elegans* embryos to form the anterior lumen. Anterior morphogenesis involves three cell types: epidermal, pharyngeal and neuroblast (neuronal precursor) cells. The development of complex structures is challenging to study *in vivo*, and our knowledge of how cells from different tissues are coordinated is limited. Using different imaging techniques, we describe the previously uncharacterized movements and patterns of the epidermal, pharyngeal and neuroblast cells that contribute to anterior lumen formation. We found that 6 specialized pharyngeal (arcade) cells at the anterior form a bulb-like shape to give rise to a stable rosette. Their projections extend to the anterior/ventral surface and express the polarity regulator PAR-6, which forms a bright focal point that marks the site of the future lumen. These projections could provide cues to guide the neuroblast and/or epidermal cells to properly position the lumen. In support of this, the mild disruption of epidermal cell fate by weak ELT-1 RNAi caused the mouth to be mispositioned. We also found that subsets of neuroblasts expressing PAR-6 assemble into two pentagon patterns that flank the bright focal point. Disrupting the neuroblast patterns by depleting ani-1 (anillin; cytokinesis regulator required for neuroblast cell division) caused delays in epidermal cell migration. Loss of one or both pentagons resulted in severe delays, and prevented epithelialization of the anterior pharynx. We propose that cues associated with these neuroblasts guide migration of the anterior epidermal cells. TIRF imaging of epidermal-specific fluorescent probes for F-actin revealed the extension of highly dynamic actin-rich projections, which are tightly coordinated with the neuroblasts. As the epidermal cells reach the anterior, PAR-6 foci form at contact points between the leading edge of the epidermal cells and the underlying neuroblasts. We are characterizing the changes in these projections that arise upon perturbation of the neuroblasts vs. pharyngeal cells, to narrow down which chemical and/or mechanical cues could be involved in mediating their migration. This work provides new insights on *C. elegans* anterior development and anterior lumen formation due to multi-tissue cooperation.

P703

**An adhesion code ensures robust pattern formation during tissue morphogenesis**

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An outstanding question in embryo development is how spatial patterns are formed robustly, despite large scale cellular re-arrangement required during tissue morphogenesis. In the zebrafish spinal cord, thirteen distinct cell types are specified along the ventral-to-dorsal axis to form stripe-like patterns, under the instruction of opposing gradients of Shh and BMP/Wnt. The Shh signal is noisy, resulting in cells specified in a mixed pattern initially. In addition, concurrent morphogenetic movement could further disrupt patterns. Nevertheless, the stereotypic stripe-like patterns still form reproducibly, raising the question of what additional mechanisms can ensure this patterning robustness. Here we set out to test if differential adhesion can assist patterning robustness during zebrafish spinal cord development.

We developed two cell-based mechanical assays based on micropipette aspiration to measure adhesion forces and adhesion preferences among three types of neural progenitors (p3, pMN, and p0 cells). Interestingly, each cell type exhibited preference to stabilize homotypic contact and adhered more strongly to cells of the same type. Subsequent genetic analyses revealed three adhesion molecules (N-cadherin, Cadherin 11, and Protocadherin 19) that are differentially expressed among the three cell types, forming a three-molecule adhesion code. When the adhesion code is perturbed, the adhesion preference to cells of the same type is lost, and the neural progenitor pattern in the spinal cord is disrupted in vivo. We discovered that both the cell fate and adhesion code are co-regulated by the common upstream morphogen signal Shh, formally connecting the morphogen gradient model with the differential adhesion model. We propose that robust patterning in tissues undergoing morphogenesis results from a previously unappreciated interplay between morphogen gradient-based patterning and adhesion-based self-organization.

P704

Cul3 is required for normal mammary gland development
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Cul3 is an E3 ligase that facilitates targeted degradation of a number of proteins involved in various cellular processes, including cell division. Disruption of Cul3 is known to affect several physiological processes and is involved in diseases including hypertension, cancer, and autism. Using a Cre-Lox targeting system, we downregulated Cul3 expression in the mouse mammary gland in order to determine the importance of Cul3 in mammary gland development. A number of phenotypic changes were observed in Cul3-deficient mammary glands, including slower outgrowth of the ductal tree, enlarged terminal end buds, and dilation of the ducts. A decrease in the amount of mammary ductal branching was noted in Cul3-deficient animals compared to wild type mice at 3 months of age. Despite the phenotypic changes to Cul3-deficient mammary glands, mice were able to nurse their litters; however, some animals showed more pronounced evidence of lactation in some mammary glands compared to others. Cul3-deficient mammary glands also showed a slight delay in the process of involution. We conclude that while Cul3 is necessary for normal mammary gland development and architecture, it is not required for lactation.
Coordinated regulation of the actin cytoskeleton, cell shape, and chamber morphology in the zebrafish heart

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During early stages of heart development, the linear heart tube (LHT) expands to create cardiac chambers with characteristic curvatures: a convex outer curvature (OC) and a concave inner curvature (IC). This stereotypical chamber shape facilitates the effective function of the embryonic heart, and errors in chamber morphogenesis are frequently associated with congenital heart disease. Using zebrafish as a model organism to understand chamber curvature formation, we have previously reported that regional changes in cardiomyocyte (CM) morphologies underlie curvature formation: as the ventricle emerges, the apical surfaces of OC cells enlarge and elongate, whereas the apical surfaces of IC cells remain relatively small and circular, similar to LHT cells. However, the subcellular mechanisms that regulate these cellular behaviors remain elusive. Recent studies have added new layers to our model for curvature formation: whereas OC cells remain relatively flat, similar to LHT cells, IC cells extend along their apicobasal (AB) axis, resulting in a cuboidal shape that is slightly constricted at the apical surface. Coupled with these differences in cell morphology, we find distinct organization of the actin cytoskeleton in each curvature: whereas F-actin is enriched in the basal domain of OC cells, it expands apically in IC cells, suggesting a link between actin dynamics and patterns of cell shape change. Consistent with this idea, we have found that inhibition of actin polymerization disrupts curvature-specific CM shapes. Synthesizing these data with prior studies, we hypothesize that ventricular curvature formation involves 1) acquisition of cuboidal (IC) or squamous (OC) morphology, and 2) curvature-specific reorganization of F-actin. Finally, we find that curvature-specific F-actin organization and CM morphologies both require the function of transcription factor Tbx5a, an established regulator of curvature-specific gene programs. Using scRNA-seq, we are working to define patterns of differential gene expression that distinguish OC and IC cells and to evaluate which of these differentially expressed genes are regulated by Tbx5a. These and other ongoing studies aim to illuminate the molecular mechanisms that connect Tbx5a gene regulation with actin cytoskeleton organization and the acquisition of OC and IC cell morphologies. Altogether, this work provides a new model for how localized regulation of actin dynamics creates patterns of CM morphologies that underlie chamber curvature formation, and may shed light on mechanisms through which chamber development goes awry in the context of congenital heart disease.

Drosophila Chitinase-like Proteins (CLPs): Imaginal disc growth factors (Idgfs) regulate tissue morphogenesis and CO₂ response

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Chitinase-like proteins (CLPs) are enzymatically inactive members of the family 18 glycosyl hydrolases. A mutation in the enzyme's catalytic site, conserved in vertebrates and invertebrates, allowed CLPs to evolve independently with functions that do not require chitinase activity. CLPs function during inflammatory responses, wound healing, and host defense, but when they persist at excessive levels at sites of chronic inflammation and tissue remodeling disorders, they correlate positively with disease
progression and poor prognosis. Research has focused on the expression patterns of CLPs and their relevance as potential biomarkers of disease or as targets for therapy, but little is known about their physiologic function. *Drosophila melanogaster* has six CLPs, termed Imaginal disc growth factors (Idgfs), encoded by *Idgf1*, *Idgf2*, *Idgf3*, *Idgf4*, *Idgf5*, *Idgf6*. In this study we focused on characterizing the physiological roles of the Idgfs by deleting each of the Idgfs using the CRISPR/Cas9 system and assessing loss-of-function phenotypes. Flies lacking all six *Idgf* have low viability and fertility. Germ cells form in reduced numbers and most are lost before reaching the gonad. Larvae have disorganized denticle belts, and adult flies have abdominal cuticle defects and ectopic wing veins. Dark cuticle patches appear on the abdomen, indicative of melanotic clots, which normally occur at sites of wounds or pathogen invasion. Eggs laid by sextuple mutants display defects in dorsal-appendage morphology, the frequency and severity of which increase upon exposure to CO2. Given the parallels between Idgfs and their human orthologs, conserved disease pathways, and sophisticated genetic tools, *Drosophila* is an excellent *in vivo* model for characterizing CLP/Idgf function. By defining the normal molecular mechanisms of CLPs, we will be able to understand the deviations that occur to tip the balance from a physiological to a pathological state. Funding: NIH grant R01 GM079433

P707

**Anchoring of cortical actin pools by the late endocytic pathway during subcellular tube guidance**

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The actin cytoskeleton participates in a wide range of cellular processes, from endocytosis to cell migration. Nucleation and anchoring at different subcellular locations allows cells to undergo cell shape changes that are important for morphogenesis. Terminal cells of the Drosophila respiratory system form a subcellular tube by invaginating the apical plasma membrane, and at least three distinct actin pools are involved in regulating their development: In the basal domain (1), in filopodia (2), and in the apical membrane domain (3). Disrupting any of these pools affects subcellular tube guidance, but why is this and what mediates the crosstalk among them is not known. We report here that actin also assembles around vesicles of the late endocytic pathway, which are mostly present towards the tip of the growing subcellular tube. We use live imaging to show that actin bundles bridge the apical and basal membranes through the late endosomes at the tip. Disturbing late endosome formation or actin nucleation at late endocytic compartments (using genetic constructs or laser ablations) affects the directionality of tube growth, uncoupling it from the direction of cell elongation. These findings highlight a role for late endosomes in regulating cell morphogenesis by organizing the actin cytoskeleton, besides their conventional role in membrane and protein trafficking.

**Dynamics of Focal Adhesions and Invadosomes**

P708

**In vivo Analysis of Focal Adhesion like Structures During Single Cell Migration**

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How does the in vivo environment dictate cell-matrix interactions during single cell migration? Although in vitro studies provide valuable mechanistic knowledge, it is critical to understand how cells migrate in their native environment in which the mechanics, signaling, and cellular interactions are intact. Focal adhesion (FA) complexes are recognized as important structures that physically link the cytoskeleton of a cell to the extracellular matrix, thus facilitating cell migration. We developed a zebrafish transplantation model in which we can efficiently visualize FA-based cell migration in vivo. This model takes advantage of the optically transparent zebrafish larvae, and the highly migratory zebrafish melanoma (ZMEL) cells, allowing for same species transplantation approaches. Strikingly, the transplanted ZMEL cells disseminate to the zebrafish skin, and form Paxillin or Ena/VASP-positive punctate structures at ventral surfaces of cells that directly contact the native matrix in the skin. Paxillin and Ena/VASP both localize to FAs in cultured cells; thus, to determine whether these structures are bona fide FAs in vivo, we performed transmission electron microscopy and detected electron dense regions that resemble adhesive structures at regions where ZMEL cells make physical contact with the matrix of the skin. Furthermore, using a collagen transgenic reporter, we visualized rearrangement of collagen fibers during ZMEL cells migration, suggesting that ZMEL cells transduce force to the surrounding matrix. We next sought to use this in vivo system to determine whether FAs form and are regulated differently in their native environment. Differences in protein dynamics can reflect distinct function in cells, thus we performed lifetime and FRAP analyses of FA proteins in vivo and compared them to metrics quantified in vitro. We found that Paxillin exhibits higher molecular turnover in vivo, as well as a faster assembly rate and a slower disassembly rate than in vitro. Due to the observed difference in Paxillin dynamics, we further tested Paxillin regulation by examining Paxillin Tyrosine118 phosphorylation (pY118) status, as it has been shown to be an important residue regulating FA turnover. As expected, pY118-Paxillin localized to FAs in ZMEL cells in culture. Surprisingly, migrating ZMEL cells in vivo did not have detectable pY118-Paxillin, suggesting differential FA regulation in vivo. We are currently further assessing Paxillin regulation and testing the functions of FA formation in a spatial and temporal manner. These studies will provide significant insight into FA-based cell migration in vivo and will aid in a better understanding of melanoma metastasis.

P709

ARHGAP17, a Cdc42-specific GAP, localizes to invadopodia and regulates their turnover
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Cancer cells can invade through tissues and metastasize by forming actin-rich membrane protrusions called invadopodia. The formation of invadopodia involves a dramatic rearrangement of the actin cytoskeleton, a process that is regulated by the Rho family of small GTPases. Rho GTPases cycle between an inactive GDP-bound and an active GTP-bound form. RhoGEFs (guanine nucleotide exchange factors) catalyze the activation of Rho proteins, whereas RhoGAPs (GTPase activating proteins) mediate their inactivation. Since RhoGAPs play a critical role in the termination of signal transduction, mutations in genes encoding RhoGAPs have drastic consequences and underlie several human diseases including cancer. However, compared with RhoGEFs, which have been studied more extensively, there is significantly less known about RhoGAPs, especially regarding their role in cancer progression and metastasis. Here, we have identified a RhoGAP, ARHGAP17, as a potential key regulator of invadopodia in triple negative breast cancer. Our results show that ARHGAP17 localizes to the invadopodia ring structure and negatively regulates their turnover by decreasing their lifetime. In these cells we have
found that ARHGAP17 has GAP specificity for the Rho GTPase Cdc42. We also show that ARHGAP17 is phosphorylated at tyrosine residues and that phosphorylation may play a role in regulating ARHGAP17 function in suppressing invadopodia formation. Based on our results we propose that ARHGAP17 is targeted to invadopodia where it promotes invadopodia disassembly by locally inactivating Cdc42.

P710

Dynamic kindlin-2 complexes containing a laminin-binding integrin are responsive to hypoxia
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The laminin-binding integrins are mechanosensory receptors critical for cell adhesion and structural organization that link the extracellular matrix (ECM) to the cytoskeleton. Integrin α6β1 is associated with prostate cancer (PCA) invasion, metastasis and decreased cancer-specific survival. Kindlins are integrin adaptors and mechanosensory focal adhesion (FA) proteins that activate and cluster integrins in response to structural ECM alterations in the tumor microenvironment. Hypoxia is a well-known inducer of tumor migration, results in ECM remodeling and is physiologically relevant for PCa. The study’s objective was to determine if integrin-kindlin-2 adhesion complexes were responsive to hypoxia and the dynamic distribution of integrin-kindlin-2 complexes using 3D immunofluorescence microscopy. The methods included a combination of 3D imaging, biochemical analysis of kindlin-2 adhesion complexes, qRT-PCR and immunocytochemistry. DU145 cells were analyzed in HEPES-containing media with 10% FBS under hypoxic conditions (1% oxygen) continuously exposed for 4, 8, 12, and 16 hours. The results showed that hypoxia increased the mRNA expression of CAIX, a well-known HIF-1α target gene, within 4 hours, resulting in 7-fold maximum expression. Similarly, VEGF-A mRNA was responsive to hypoxia as expected. In contrast, HIF-1α, α6 integrin, and kindlin-2 mRNA levels remained unchanged as compared to normoxia. Strikingly, we found that kindlin-2-containing adhesion complexes increased under hypoxia conditions as compared to normoxia and detected by immunoprecipitation. The complexes contained both α6β1 and α5β1 integrins. It is important to note that the constitutive levels of integrin were not altered by hypoxia. Using immunofluorescence microscopy (IFM) under normoxic conditions, we confirmed the location of kindlin-2 within phosphorylated (Y31) paxillin (pPAX)-containing FAs and within extended plasma membrane domains exclusive of pPAX. However, under hypoxic conditions, an increased reorganization of pPAX-containing kindlin-2 complexes occurred within 4 hours, was continuously changing up to 16 hours, with increasing fibrillar forms of FAs. In addition, kindlin-2 was observed as increased in plasma membrane protrusions devoid pPAX. The dynamic nature of the hypoxia-driven FAs was observed by a time course analysis and indicated the apparent assembly and disassembly of the structures during 16-hours of exposure to hypoxia. The results were independent of cellular toxicity. We conclude that kindlin-2-integrin complexes are responsive to hypoxia and contain both, the expected α5β1 integrin in FAs, and the unexpected α6β1 integrin in the membrane exclusive of FAs. (Supported by NIH grants CA P30 23074, DOD W81XWH-19-1-0455, and NCI R01 CA242226).
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**Retrograde actin flow-associated motions of focal adhesion molecules visualized by single-molecule speckle (SiMS) microscopy**

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Cell migration is important for many biological processes including development, wound healing and tumor metastasis. At the cell leading edge, the retrograde actin flow, continuous centripetal movement of the actin network, is widely observed in migrating cells. During cell migration, linkage between the retrograde actin flow network and focal adhesions promotes cell protrusion as postulated in the clutch model (Mitchison and Kirschner, Neuron, 1988). However, how focal adhesion molecules link to the actin network moving along the retrograde flow remains unclear. Single-Molecule Speckle (SiMS) microscopy is a powerful approach to directly monitor the mechanics linking actin dynamics and cell adhesion at the molecular level. Our previous fluorescence single-molecule actin speckle analysis revealed that the retrograde actin flow speeds in and outside nascent adhesions showed no significant difference, indicating that actin filaments in lamellipodia flow uniformly even within nascent adhesions (Yamashiro et al. MBoC 25:1010, 2014). How can focal adhesion molecules link to the actin filaments that continuously shift ~10 subunits/sec? In this study, we examined molecular motions of focal adhesion components and actin in lamellipodia by using SiMS microscopy. Several focal adhesion components including Talin exhibit flow-associated motion along the retrograde actin flow in addition to stationary state. Interestingly, focal adhesion components exhibit dynamic transition between the two states. Our findings provide new insights into the interaction between the retrograde actin flow and focal adhesion molecules at the cell leading edge.

P712

**Delineating the role of p27\(^{kip1}\) and the cell cycle in invadopodia-mediated invasion**

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Tumor cell invasion comprises cell migration and assembly of invadopodia, which are protrusions capable of degrading the extracellular matrix (ECM). The effect of cell cycle progression on invadopodia-mediated invasion has not been well described. Previous studies from our group revealed that invadopodia function is enhanced in the G1 phase of the cell cycle in breast carcinoma MDA-MB-231 cells. Moreover, we and others showed that the cytoplasmic pool of cyclin-dependent kinase inhibitor (CKI) p27\(^{kip1}\) localizes to the sites of invadopodia assembly. A recent study revealed that p27\(^{kip1}\) physically interacts with cortactin, a core protein of the complex, and triggers the disassembly of the complex. In this study, we further investigate the molecular mechanism and the dynamics of the interaction between p27\(^{kip1}\) and the invadosome complex. Proximity ligation assay (PLA) and co-IP experiments indicate that p27\(^{kip1}\) physically interacts with Tks5, the unique member of the invadosome complex. Interestingly, when cells were synchronized in G1, the extent of the interaction (the number of PLA puncta) decreased, while the number of PLA puncta increased when cells were synchronized in S phase. It appears that the presence of p27\(^{kip1}\) at the site of invadopodia assembly is inversely correlated with
the ability of cells to degrade, consistent with its role in the disassembly of the invadosome complex. These findings warrant further investigation into determining the exact sequence and timing of p27kip1 recruitment to the invadosome complex and how this interaction is linked to cell cycle progression.

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Exploring the role and regulation of PKA in focal adhesions, a critical subcellular compartment in cell migration.

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The cAMP-dependent protein kinase (PKA) binds to a variety of A-kinase anchoring proteins (AKAPs) which provides spatiotemporal regulation of PKA activity in a variety of complex cellular processes. For example, efficient cell migration requires (AKAP-mediated) localization and regulation of PKA activity within the leading edge. However, the importance of PKA activity in other cellular compartments that are important for cell migration is not fully understood. Focal adhesions (FAs) are multiprotein complexes that link extracellular matrix-bound integrins to intracellular actin filament bundles, and thus play major roles in generating traction force and transmitting extracellular signaling during cell migration. A number of observations suggest that FAs may be a direct target for localized PKA activity. Inhibition of PKA leads to morphological and positional changes in FAs during cell spreading. Furthermore, we have recently observed dynamic PKA activity within FAs, suggesting that PKA may function directly on or within FAs. To address this possibility, through a proximity-dependent biotin labeling method using miniTurbo-tagged PKA regulatory RII subunit, we have screened potential AKAPs from isolated FAs. Proteomic analyses of biotinylated proteins yielded a list of known and novel adhesion proteins with putative AKAP consensus sequences. Using a combination of biochemical and optogenetic techniques, we are currently validating a number of these hits as potential FA-AKAPs, which will provide significant new mechanistic insight into how PKA controls cell migration.

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Vimentin drives cell extension formation by controlling β1 integrin function

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Cell migration through soft connective tissues relies on cell adhesion to collagen and the formation of actin-based membrane extensions. Recent data indicate that vimentin filaments are also centrally involved in cell extension formation and migration through collagen matrices. Within the adhesions that attach cells to collagen, vimentin directly interacts with β1 integrin and paxillin. Currently, the mechanisms by which vimentin cooperates with these proteins to regulate adhesion, cell extension formation and migration through soft connective tissues are not defined. We considered that vimentin provides critical control adaptor functions that control β1 integrin binding to collagen. Here we examined whether vimentin acts as an adaptor protein that orchestrates the formation, stabilization, and turnover of β1 integrin-dependent cell adhesions to collagen in time and space, which in turn leads to cell extension formation and cell migration. We found that in fibroblasts, deletion of vimentin promotes >2-fold higher talin-dependent activation of β1 integrin (p<0.01). Loss of vimentin was also
associated with reduced (>60%; p<0.001) paxillin recruitment to focal adhesions and inhibition of β1 integrin clustering (>50%; p<0.01). The reduced paxillin recruitment was associated with diminished Cdc42 activation (>65%, p<0.001). Cdc42-related PAK1 activation also reduced vimentin phosphorylation and filament assembly, which were associated with 60% inhibition of cell extension formation (p<0.001). We propose that vimentin filaments are essential for the recruitment of paxillin into adhesion complexes and for activation of Cdc42 and PAK1, which are required for the formation of cell extensions. Moreover, these processes are ultimately dependent on vimentin phosphorylation, which is mediated by PAK1. Vimentin phosphorylation in turn promotes the maturation of vimentin filaments, which is essential for the recruitment of β1 integrin regulatory proteins to adhesion complexes and the regulation of β1 integrin activation and affinity. As these processes are essential for tissue regeneration and repair in physiological remodeling of connective tissues and wound healing, our current view of the functions of vimentin in fibroblasts may need to be expanded to include a central role in regulating the signals that control the formation and function of cell adhesions.

Epigenetics and Chromatin Remodeling

P715

Chromatin compaction and architecture changes with cellular quiescence

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Quiescence is the reversible arrest of cell proliferation that is important across biological processes like stem cell maintenance and cancer cell dormancy. We and others have shown that transition to quiescence lead to specific upregulation of the H4K20me3 mark (by ~10-fold) and global changes in chromatin organization. How the 4D organization of the genome is regulated in quiescence is not known in detail. To determine the levels of factors regulating these nuclear events, we used a quiescence cell culture model based on human dermal fibroblasts. Proliferating (P) cells were introduced into quiescence by 7-days of serum-starvation (SS) or contact inhibition (CI) and then restimulated to a proliferating state for 24 hours (SS-R or CI-R). By performing stranded RNA-seq on the above five conditions (P, CI, SS, SS-R, and CI-R) we found that specific readers of H4K20me3, such as the Spindlin family of proteins, are strongly upregulated (~4 fold) in quiescence. When cells were restimulated to enter the cell cycle (SS-R or CI-R), the levels of these readers returned to proliferating levels. This suggests that the effects of H4K20me3 on quiescence are mediated through these readers. Similarly, RNA-seq data show significant downregulation of the members of condensin I and II and cohesin complexes with quiescence. In SS-R and CI-R cells, the expression levels of these genes return to the level of P cells. These results have been validated by western blot analysis of chromatin extracts. Our quiescence time series experiments revealed that chromatin-associated condensin levels drop drastically at distinct points during quiescence entry but are re-expressed as cells return to the cell cycle. These findings suggest that chromatin remodeling proteins and chromatin architecture are changing in quiescence and play an important role in the transitions into and out of quiescence. Additionally, our flow cytometry data shows that in quiescence there is a 22 percent increase in the number of cells with small nuclei and more compact chromatin, when compared to P cells with the same DNA content. When restimulated, there is a 26 percent decrease in the number of cells with small nuclei, when compared to
SS cells. By microscopy, we observe that the nuclei are 36% narrower in quiescent cells than in proliferating cells—quantified by the ratio of longest diameter to shortest diameter of the nuclei. Like our previous data, this change in nuclear shape is reversed when cells are restimulated. Taken together, our data suggest an important link between chromatin-associated events and quiescence that will provide fundamental understanding to the mechanisms of reversible cell-cycle exit and 4D genome organization.

P716

**Determining the mechanism of Set1-mediated telomere regulation in* Saccharomyces cerevisiae***

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Telomeres are nucleoprotein structures found at the ends of linear chromosomes which function to counteract telomere shortening during replication as well as prevent DNA ends from being treated as double-stranded breaks. Proper telomere dynamics are a necessary component in the maintenance of genomic stability, and dysfunction in this system is associated with cellular aging and many types of cancer. The yeast protein Set1 is an H3K4 methyltransferase that has been implicated in several genomic functions, including regulation of gene expression, the DNA damage response, and telomere length maintenance. Although a role for Set1 at telomeres has been observed, the mechanism through which Set1 regulates telomeres is still largely unclear. In previous work, we found that the transcriptomes of cells lacking Set1 are highly correlated with transcriptomes belonging to mutants that display defects in telomere maintenance, demonstrating a specific role for Set1 in pathways required for telomere integrity, in addition to its regulation of sub-telomeric silencing. In our recent work, we have found that cells lacking both telomerase and Set1 display an enhanced senescence phenotype and have observed altered expression and localization patterns of some telomere-bound proteins in the absence of Set1, emphasizing the presence of telomere defects in the set1Δ. In order to determine the contributions of both the catalytic and non-catalytic activities of Set1 to the set1Δ telomere phenotypes, we have analyzed several Set1 and H3K4 methylation-defective mutants and have determined that Set1’s role in sub-telomeric silencing, telomere length maintenance, and the DNA damage response are likely both H3K4 methylation dependent and independent, but are independent of Set1’s RNA-binding function. Overall, our data supports a model in which methylation by Set1 promotes telomere health through the regulation of known telomere maintenance proteins.

P717

**Drosophila Keap1 and Nrf2 oxidative/xenobiotic response factors cooperatively regulate heterochromatin and developmental transcription***

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How environmental toxins regulate epigenetic status and developmental programs is poorly understood at the molecular level. The Nrf2 transcription factor and its key regulator Keap1 form a major pathway that mediates transcriptional responses to oxidative and xenobiotic stimuli. Mutations in Nrf2 and Keap1 are highly related with many human diseases, including cancer. We are exploring the novel molecular mechanisms and biological functions of dKeap1 and CncC, the Drosophila homologues of Keap1 and Nrf2. We have revealed a novel developmental function of dKeap1 and CncC in the regulation
of ecdysone signaling and metamorphosis. In addition, both dKeap1 and CncC are required for the maintenance of heterochromatic silencing, providing the first evidence in support of their epigenetic roles in chromatin remodeling. Interestingly, these novel functions of dKeap1-CncC complex are related with a non-classic mechanism in which dKeap1 binds chromatin and cooperates with CncC. This is in contrast with the conventional model stating that Keap1 inhibits Nrf2 activity through interacting and degrading Nrf2 in cytoplasm. Therefore, dKeap1-CncC complexes likely regulate detoxifying genes and developmental genes using different molecular mechanisms, respectively. To fully explore the biological functions of Keap1-Nrf2 family proteins, we employed polytene chromosome BiFC (bimolecular fluorescence complementation) assay to visualize the genomic loci that are exclusively targeted by dKeap1-CncC complex and map the developmental genes that are co-activated by dKeap1 and CncC. In addition, a mutant that specifically abolishes the chromatin-binding of dKeap1 while retains its ability to inhibit CncC was generated, allowing us to characterize the developmental functions of dKeap1-CncC that are independent of their classic role in redox-control. These studies are expected to reveal novel mechanisms whereby stress response factors mediate the epigenetic and developmental adaptations to environmental toxins.

P718

**Visualization of DNA methylation and its mediators at the single molecule level**

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Cells can achieve different outputs from the same genome by utilizing DNA methylation to regulate gene expression. In mammals, methylated CpG dinucleotides recruit DNA “reader” proteins. Once bound, reader proteins can act as scaffolds to recruit downstream factors that mediate transcription and other genomic processes. Studying the binding specificity of different readers will help us understand how cells can interpret a unique distribution of DNA methylation to establish and maintain distinct cellular identities. We developed a novel, microfluidic assay which can be used to probe DNA methylation patterning and recognition at the single-molecule level. Using fluorescently-labeled reader proteins and CRISPR/dCas9-based technology, we can visualize a distribution of DNA methylation across kilobase pairs of DNA at nucleotide resolution. With this system, we show that reader binding is dependent on additional genomic cues beyond the mere presence of a methylated CpG dinucleotide. We can leverage this system to identify how these signals lead to unique binding distributions of individual readers across the genome. Using this experimental framework, we will begin characterizing how additional molecular players regulate the dynamics of DNA methylation.

P719

**A novel histone modification mediates DNA accessibility with innate immune activation**

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Protein tyrosine nitration (PTN) is a post-translational modification that has been reported in numerous cardiovascular diseases resulting from oxidative and nitrosative stress. PTN is known to alter cellular and metabolic processes that can result in either a gain (e.g. fibrinogen) or loss (e.g. MN-SOD) of function.
We have previously shown that nuclear reprogramming (e.g. of fibroblasts to endothelial cells, or to induced pluripotent stem cells) requires innate immune activation (Lee et al., 2012; Meng et al., 2016). We find that during this process, inducible nitric oxide synthase (iNOS) translocates to the nucleus, where it binds to and modifies the activity of epigenetic modifiers, to promote DNA accessibility. We propose that histone tyrosine nitration (HTN), mediated by iNOS, is required by histones for chromatin remodeling during transdifferentiation of fibroblast to endothelial cells undergoing transflammation. To examine the role of HTN during transflammation BJ fibroblasts were treated for 3 days with 30 ng/mL poly I:C to activate innate immunity. Cells were collected for immunoprecipitation by nitrotyrosine and immunoblotted for histones H2A, H2B, H3, and H4. Cells were stained with iNOS, nitrotyrosine, and DAPI, then imaged on a FV3000 confocal microscope. Molecular dynamic simulations and mass spectrometry (MS) were completed in collaboration with The University of South Florida. Sequential MS were conducted at Baylor College of Medicine. Preliminary mass spectrometry results identified tyrosine nitration on Histone 3 tyrosine 41 (H3Y41(nitro)). Further analysis by molecular dynamic simulation predicted a more open chromatin configuration of DNA upon H3Y41(nitro). On day 3 of the transdifferentiation, BJ fibroblasts treated with poly I:C show an increase of HTN of histones H2A, H3 and H4, whereas H2B remained unchanged. These data were further supported by successive MS analysis of the remaining IP sample. Lastly, we observe co-localization of iNOS to sites of tyrosine nitration following poly I:C treatment, suggesting a possible role for iNOS in the nitration of histone proteins. To conclude, we have previously demonstrated that iNOS plays a role in DNA accessibility during nuclear reprogramming and cell fate changes. We now show evidence for HTN during the transdifferentiation of BJ fibroblasts that coincides with a more open chromatin state following innate immune activation during transflammation. HTN is observed to be co-localized with iNOS in the nuclear region as a result of poly I:C treatment. These observations are consistent with our hypothesis that HTN, mediated by iNOS, occurs during activation of innate immune signaling, and may regulate chromatin structure and DNA accessibility during transdifferentiation.

P720

**Essential Roles of Polyhomeotic Homolog 1 (Phc1) in Early Retinal Development**

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The eye is formed by multiple cell types, and its development is regulated by a number of steps coordinated with both extrinsic and intrinsic signals. Uncommitted stem cells undergo spontaneous differentiation in response to locally or distally produced signaling molecules. Malformation or aplasia of the eye greatly impacts systemic activities; thus, understanding the mechanisms for retinal differentiation is critical from both basic science and clinical point of view. Polyhomeotic homolog 1 (PHC1), also known as RAE28, is a member of the polycomb group (PcG) proteins that is thought to modulate gene silencing via chromatin modification. In previous studies, the deficiency of the Phc1 gene has been shown to cause eye malformations and perinatal mortality. However, the detailed mechanisms by which Phc1 regulates eye development remain to be elucidated. The objective of this study was to investigate the roles of Phc1 during eye development. For this purpose, Phc1-knockout (Phc1-KO) mouse embryonic stem cells were generated using the CRISPR/Cas9 system, then either wild-type or Phc1-KO cells were differentiated into retinal organoids. Both genotypes of cells successfully differentiated into SOX2-positive neural progenitors; however, early retinal progenitor marker proteins, including PAX6 and
RX, failed to be expressed in the Phc1-KO cells during retinal differentiation. Additionally, high-throughput expression analyses identified genes such as Bmp4 and Aldh1a2 with altered expression levels in the absence of Phc1. Furthermore, to elucidate the cell or tissue extrinsic cues involved in retinal development, a mouse line devoid of the Phc1 gene was generated. In homozygous knockout embryos, the optic cup morphogenesis was severely perturbed at embryonic day 10.5, with failed invagination of the lens ectoderm, while whole-brain development remained largely normal. These results suggest that the dorsal-ventral polarity and the differentiation state were altered in the Phc1-KO mice. Taken together, both in vitro and in vivo analyses revealed that PHC1 is essential for the early stages of retinal differentiation. Identifying target genes directly regulated by PHC1 will be critical to further understand the role of PHC1 in retinogenesis.

Establishing and Maintaining Organelle Structure: Nucleus and Nucleoskeleton

P721

The properties of membraneless organelles are adapted to local conditions in a free living fungi.
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How cells pattern and compartmentalize their cytoplasm in space and time is a critical subject in biology. Work in the last decade has found that cells rely on membraneless organelles as a critical method of compartmentalization. These organelles are not separated from the rest of the cell by a lipid bilayer, instead they are formed through Liquid-Liquid Phase Separation (LLPS) in which a well-mixed solution spontaneously demixes into a concentrated droplet phase and a dilute bulk phase. These droplets have specific properties such as viscosity, pore size, yet how these properties relate to their function and how they are controlled is not well understood. In the free-living filamentous synicital fungus Ashbya gossypii asynchronous nuclear division in the hyphae requires the formation of droplets containing the protein Whi3 and the cyclin RNA CLN3. Whi3 also forms droplets with the polarity RNAs BNI1 and SPA2. These droplets regulate the ability of the hyphae to branch. Like other phase-separating proteins Whi3 is structured to promote multivalent interactions through an RNA recognition motif (RRM) that and a disordered domain rich in glutamine (polyQ). Loss of either of these domains results in failure to form droplets. Because LLPS is a thermodynamic process, temperature plays a role in determining whether phase separation happens and influences the properties of the resulting droplets. Free living fungi are exposed to the ambient temperature and therefore are under selective pressure to adapt to their local climate. We hypothesized that if the material properties of droplets are important then they should be optimized for a given climate. Using a collection of fifty wild-isolated Ashbya from different climatic regions in the United States we tested the effects of temperature on Whi3 droplets. We found that the interbranch distance, which is controlled by membraneless organelles, varied between isolates when grown at high or low temperature as did the synchronicity of the cell cycle. Using whole genome sequencing we found differences in the primary sequence of phase separating components. We then tested these primary sequences differences using an in vitro phase separation assay and molecular dynamic simulations and found protein domains and RNA motifs critical for temperature dependent properties. We also show that these domains are critical for droplet function in vivo. Together this work indicates ways that membraneless organelles are adapted to their native climate and how these adaptations affect function.
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The KASH-independent role of ANC-1 in organelle positioning in C. elegans

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KASH proteins in the outer nuclear membrane interact with SUN proteins to form LINC complexes that connect nuclei to the cytoskeleton. In Caenorhabditis elegans, the Nesprin-1/2 ortholog ANC-1 is proposed to physically tether nuclei to the actin cytoskeleton through its C-terminal KASH domain and the N-terminal calponin homology (CH) actin-binding domains. However, deletion of the CH domains caused no nuclear positioning defects, and disruption of SUN/KASH interaction only caused mild nuclear positioning defects. ANC-1 also contains spectrin-like regions with unknown function. We hypothesized that the central spectrin-like repeats of ANC-1 are essential for nuclear anchorage. We observed strong nuclear anchorage defects after deleting the spectrin-like region. Interestingly, the deletion of the transmembrane domain adjacent to the luminal KASH peptide enhanced the nuclear anchorage defects of the KASH deletion mutant. ANC-1 is localized to the ER and the nuclear envelope. In anc-1 null mutants, the ER, mitochondria, and lipid-droplets were all unanchored and sometimes moved throughout the cytoplasm. Without the spectrin-like region, ANC-1 is enriched on the nuclear envelope and the ER structure is disrupted. We propose a cytoplasmic integrity model where ANC-1 localizes to the ER and functions in positioning nuclei, ER, mitochondria, and likely other organelles in place.

P723

The lipin1 phosphatase CTDNEP1 links control of ER membrane biogenesis via mTOR to formation of micronuclei

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After open mitosis, membranes derived from the endoplasmic reticulum (ER) wrap around segregated chromosome masses to form the nuclear envelope (NE). Micronuclei, a common feature of cancer cells, form from ER-derived membranes that wrap around single chromosomes that are not incorporated into the main chromatin mass. The mTOR kinase, which is commonly amplified in cancer, regulates the synthesis of lipids that generate the membranes of the ER/NE through phosphorylation of lipin1 phosphatidic acid phosphatase. Despite the importance of mTOR and micronuclei to cancer, a link between misregulated mTOR signaling and formation of micronuclei has not been made. Here, we show that the protein phosphatase CTDNEP1 antagonizes mTOR-mediated phosphorylation and subsequent proteasomal degradation of lipin1 to limit micronuclei formation. In U2OS cells deleted for CTDNEP1, lipin1 is hyperphosphorylated and highly unstable leading to an increase in the rate of de novo fatty acid synthesis, excess ER membrane biogenesis and micronucleation. The increase in micronuclei in CTDNEP1-deleted cells corresponds to an increase in the percentage of mitotic cells with lagging chromosomes. In early phases of mitosis, excess ER membranes infiltrate aligning chromosomes and also appear to confine the mitotic spindle, suggesting that ER expansion causes mitotic defects. Consistent with this, release from spindle microtubule-destabilizing drugs in CTDNEP1-deleted cells results in severe spindle morphology defects and impedes the gathering of chromosomes into a major
nuclear compartment - sets and single chromosomes remain physically separated from each other, resulting in severe micronuclealation. Inhibition of de novo fatty acid synthesis by small molecule inhibition of acetyl CoA carboxylase or expression of a mTOR-phosphodeficient, catalytically active lipin1 prevents the expansion of ER membranes and formation of micronuclei in CTDNEP1-deleted cells. Thus, CTDNEP1 limits the rate of de novo fatty acid synthesis by regulating lipin1 protein stability to restrict ER membrane biogenesis and prevent the formation of micronuclei. We identify cancer-associated inactivating mutations in CTDNEP1 that together with these data suggest that the upregulation of de novo fatty acid synthesis in the context of misregulated mTOR signaling and/or loss of CTDNEP1 activity leads to the formation of micronuclei.

P724

The N-terminal Region of Jaw1 Has a Role to Inhibit the Formation of Organized Smooth Endoplasmic Reticulum as an Intrinsically Disordered Region

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Jaw1/LRMP is a type II integral membrane protein that is localized at the endoplasmic reticulum (ER) and outer nuclear membrane. Previously, we reported the function of Jaw1 in maintaining nuclear shape as a KASH protein via its carboxyl terminal region, a component of the linker of nucleoskeleton and cytoskeleton complex in the oligomeric state. Although the oligomerization of some KASH proteins via the cytosolic region serves to stabilize protein-protein interactions, the issue of whether or not the oligomerization of Jaw1 is via cytosolic region and how this process is regulated is not completely understood. Therefore, we attempted to characterize the role of three distinct regions on the cytosolic face of Jaw1: the N-terminal region (44-183 aa), the coiled-coil domain (184-333 aa), and the stem region (334-470 aa) in terms of oligomerization. First, to identify the oligomerization site of Jaw1, GFP tagged each region of Jaw1 were co-expressed with FLAG tagged full-length Jaw1 in HEK293 cells and the lysates were subjected to co-immunoprecipitation using anti-FLAG beads. Results showed that the coiled-coil domain was the main oligomerization site. Next, to evaluate the role of each region on intracellular localization of Jaw1, deletion mutants lacking each region were transfected into B16F10 cells. Results of immunostaining showed the ectopic expression of an N-terminal region deleted mutant (Jaw1 ΔN) caused the formation of abnormal ER membranous structures such as nuclear karmellae and whorls, which are referred to as organized smooth ER (OSER). Although this OSER formation were commonly observed by the expression of both human and mouse Jaw1 ΔN, the DICHOT system, a software to explore the IDR, showed that both N-terminal regions function as IDR. Furthermore, this OSER was not significantly observed in the cells that were ectopically expressing a mutant lacking the N-terminal region and the coiled-coil domain (Jaw1 ΔN Coil), which suggests that the N-terminal region prevents the disordered oligomerization of Jaw1. Finally, co-immunostaining images of the cells co-expressing Jaw1 and FLAG tagged type III inositol 1,4,5-triphosphate receptor (IP₃R3) or FLAG tagged SUN2 showed that the OSER derived from Jaw1 ΔN interferes with the localization of these interactors. In summary, this study suggests that the N-terminal region of Jaw1 inhibits OSER formation as an IDR. This prevents the disordered oligomerization due to the exposure of coiled-coil domain, an oligomerization site, to maintain the homeostatic localization of interactors on the ER membrane.
**P725**

**LEM2 phase separation promotes ESCRT-mediated nuclear envelope reformation.**

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During cell division, remodelling of the nuclear envelope enables chromosome segregation by the mitotic spindle. The reformation of sealed nuclei requires ESCRTs (endosomal sorting complexes required for transport) and LEM2, a transmembrane ESCRT adaptor. Here we show how the ability of LEM2 to condense on microtubules governs the activation of ESCRTs and coordinated spindle disassembly. The LEM motif of LEM2 binds BAF, conferring on LEM2 an affinity for chromatin, while an adjacent low-complexity domain (LCD) promotes LEM2 phase separation. A proline-arginine-rich sequence within the LCD binds to microtubules and targets condensation of LEM2 to spindle microtubules that traverse the nascent nuclear envelope. Furthermore, the winged-helix domain of LEM2 activates the ESCRT-II/ESCRT-III hybrid protein CHMP7 to form co-oligomeric rings. Disruption of these events in human cells prevented the recruitment of downstream ESCRTs, compromised spindle disassembly, and led to defects in nuclear integrity and DNA damage. We propose that during nuclear reassembly LEM2 condenses into a liquid-like phase and coassembles with CHMP7 to form a macromolecular O-ring seal at the confluence between membranes, chromatin and the spindle. The properties of LEM2 described here, and the homologous architectures of related inner nuclear membrane proteins, suggest that phase separation may contribute to other critical envelope functions, including interphase repair and chromatin organization.

**Establishment and Maintenance of Polarity**

**P726**

**How to organize a uniform oriented microtubule array in dendrite**

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A polarized arrangement of neuronal microtubule arrays is the foundation of membrane trafficking and subcellular compartmentalization. Conserved among both invertebrates and vertebrates, axons contain exclusively “plus-end-out” microtubules while dendrites contain a high percentage of “minus-end-out” microtubules, the origins of which have been a mystery. Here we show that the dendritic growth cone contains a non-centrosomal microtubule organizing center, which generates minus-end-out microtubules along outgrowing dendrites and plus-end-out microtubules in the growth cone. RAB-11-positive endosomes accumulate in this region and co-migrate with the microtubule nucleation complex γ-TuRC. The MTOC tracks the extending growth cone by kinesin-1/UNC-116-mediated endosome movements on distal plus-end-out microtubules and dynein clusters this advancing MTOC. Critically, perturbation of the function or localization of the MTOC causes reversed microtubule polarity in dendrites. These findings unveil the endosome-localized dendritic MTOC as a critical organelle for establishing axon-dendrite polarity.
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The ability of cells to polarize and move toward external stimuli plays a crucial role in development, as well as normal and pathological physiology. We have shown that during random migration and in response to chemoattractants, cells maintain dynamic complementary distributions of Ras activity and phosphatidylinositol (3,4)-bisphosphate [PI(3,4)P2]. Here, we demonstrated that there is an ongoing flow of vesicular PI(3,4)P2 through the cell and a compensatory forward flow along the membrane, which establishes a back-to-front gradient of this phosphoinositide. Previous models have suggested that vesicular and plasma membrane flows are important for cell migration, although the directions were typical reversed. Specifically, our findings show that, first, PI(3,4)P2 localizes to the lagging edge of Dictyostelium amoeba and neutrophils. Surprisingly, it also localizes to retracting leading edge protrusions and nascent macropinosomes, even in the absence of PIP3. Second, PI(3,4)P2 is internalized on macropinosomes and transported on microtubules into the cytosol. The macropinosomes break up into smaller PI(3,4)P2-enriched vesicles, which dock and fuse to the plasma membrane at the cell rear. Third, green-to-red photo-converted PI(3,4)P2 molecules at the back diffuse along the membrane towards the front, where they are degraded. Last, a stochastic mathematical model confirmed that this cycle can bring about a stable back-to-front gradient. In more polarized cells, owing to a slower arrival time of PI(3,4)P2 vesicles and a slower decay rate of PI(3,4)P2 on the membrane, the PI(3,4)P2 signal at the back broadens and extends further to the front. This surprising reverse fountain flow of PI(3,4)P2 helps establish the back-to-front gradient of the phosphoinositide and, since PI(3,4)P2 antagonizes Ras activation, cell polarity.

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Early events driving B lymphocyte polarization

B lymphocytes initiate the humoral adaptive immune response upon recognizing their cognate antigen on the surface of another cell. The B lymphocyte then form an immune synapse with the opposing cell, accumulates the antigen at the center of the contact, and reorganize its organelles to polarize, ultimately enabling antigen extraction and specific antibody production. This places polarization of the B lymphocyte at the center of its physiological function, and calls for a better understanding of this fast and global rearrangement. The process of B cell polarization is poorly understood, and it is difficult to discern whether it is a phenomenon orchestrated by a dominant element (for example the centrosome), or whether it is a general coordinated reorganization. Our goal is to identify a sequence of events in the polarization of organelles, and the role played by their interactions in the acquisition of polarity. We developed a microfluidic tool that allows us to reconstitute immune synapse formation between a B cell and an antigen-coated droplet mimicking the antigen presenting cell, in a multiplexed manner. Using quantitative live imaging, we could characterize the movement of most major actors upon antigen
recognition from the first contact. A sequence emerged from our observations, happening in ~5min: F-actin is transiently nucleated at the immune synapse, then cleared as the centrosome polarizes. Lysosomes gather around the centrosome that then detaches from the nucleus, allowing the latter to be transported away from the synapse. We are currently assessing the dependence of organelle movements on actin and microtubules, which will allow us to define organelle-organelle interactions during polarization and the role played by the cytoskeleton. Interestingly, our first results suggest a central role of microtubules in lysosome and nuclear polarization, while F-actin does not seem to impact these processes as strongly and is dispensable for centrosome polarization. By creating a new set of tools to investigate the dynamics of B cell polarization, this project allows an unprecedented systematic characterization of the polarization of organelles, and will provide a comprehensive map of organelle-cytoskeleton interactions during B cell polarization.

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Establishment of apico-basolateral polarity in the embryonic C. elegans intestinal epithelium

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Apico-basolateral polarization is essential for epithelial cells to function as selective barriers and transporters and to provide mechanical resiliency to organs. Different epithelia use divergent mechanisms to establish polarity, which are only beginning to be understood. Here, we use the C. elegans embryonic intestine to investigate how epithelial cells establish apico-basolateral polarity in vivo. PAR-3 is the most upstream known polarity regulator in this tissue, and is required to establish apical polarity at the intestinal midline (Achilleos et al 2010). We find that intestine specific depletion of PAR-3 disrupts both apical and basolateral protein localization, resulting in edematous intestines and early larval lethality. In other organisms, the actin binding protein, afadin/AFD-1, and the basolateral kinase, PAR-1, are essential for PAR-3 localization during epithelial polarity establishment. We tagged AFD-1 and PAR-1 with GFP to explore their endogenous localization and a tissue specific degron tag to determine if these proteins are required for C. elegans intestinal polarization. We find that AFD-1 and PAR-1 localize to subapical junctions in the polarizing intestine, only partially co-localizing with PAR-3. Intriguingly, these proteins localize basal to the presumptive apical surface prior to the exclusion of basolateral proteins (LET-413, LGL-1) from this surface. This pattern of localization suggests that polarity establishment occurs in a stepwise manner with the apical surface and junctions established before the basolateral domain. In contrast to other organisms, we find that intestine specific depletion of AFD-1 or PAR-1 does not affect the initial localization of PAR-3 to the apical surface. Instead, AFD-1 depletion results in subtle intestinal defects and slowed growth in later embryonic and larval stages, suggesting a possible role for AFD-1 in junction maturation or integrity. Similarly, PAR-1 depletion appears to result in an expansion of the apical surface at later embryonic stages, suggesting that while it is not required for polarity establishment, it may play a key role in maintenance.
Novel tools to investigate mammalian planar cell polarity establishment
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Planar cell polarity (PCP) is a highly conserved pathway that regulates the collective polarization of cells along a tissue plane and is fundamental for embryonic development and tissue organization. A defining feature of PCP is the asymmetric localization of its core components, which transition from uniform to asymmetric distributions at cell junctions over time. Frizzled (Fz) localizes on one side of the cell, while Vangl localizes to the other. An atypical cadherin, Celsr, localizes to both sides of the cell and forms homotypic adhesions that are predicted to aid in the interaction between Fz and Vangl across cell interfaces. It is thought that both intercellular and intracellular interactions between core PCP components drive this asymmetric partitioning and couple polarity between cells in the tissue. However, the dynamics of PCP protein polarization, especially in mammalian tissues, remain poorly understood. Previous tools to study mammalian PCP rely on either antibodies or overexpression of tagged PCP proteins. Antibody staining is not conducive to studying protein dynamics, while overexpression can disrupt PCP protein localization and function. To circumvent these issues, we used 2C-HR-CRISPR to generate three novel mice with fluorescent tags inserted into the endogenous loci of three core transmembrane PCP proteins: Fz6, Celsr1, and Vangl2. We verified the proper localization and function of the endogenously tagged proteins in the mouse embryonic epidermis, where PCP controls the orientation of thousands of hairs across the body surface. These tools allow for live imaging of PCP protein dynamics in the mouse embryonic epidermis, as well as in primary keratinocytes and organotypic skin cultures. Using these powerful tools, I will present our progress in understanding the dynamics of PCP establishment in the mouse epidermis. This work was supported by the NIH-NIAMS under award number F31-AR077407-01 to L.P.B. and R01-AR066070 to D.D.

aPKC and AGS3 promote planar spindles through NuMA phosphorylation
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Orientation of the cell division axis regulates cellular position and cell fate in tissues. Spindle positioning requires external as well as cell autonomous polarity cues but how polarity cues control spindle orientation is unclear. The polarity protein aPKC regulates in vivo spindle orientation and cell fate in the mammalian epidermis (Niessen et al., JCB, 2012). Using a minimal primary keratinocyte cell culture system with most spindles being planar oriented, we aimed at identifying how mammalian aPKCs control mitotic spindle orientation in the absence of intercellular junctional polarity cues. Inactivating both aPKCs promoted random spindles on the expense of planar spindle both in vivo and in this minimal cell culture system, thus demonstrating the validity of the model. Upon loss of aPKC the spindle orientation machinery components Gai/LGN/NuMA were no longer localized at the cortex early during mitosis. We then asked how loss of LGN and its mammalian orthologue AGS control spindle orientation. Whereas
knockdown of LGN did not alter spindle orientation, AGS knockdown increased spindle angle to a similar extend as loss of aPKC, in agreement within vivo observations (Williams et al., NCB 20XX). Interestingly, this increase in spindle angle upon loss of aPKC or AGS3 depends on LGN, as additional loss of LGN reversed this increase. We then used SILAC and mass spectrometry to ask how aPKC controls spindle positioning. This unbiased screen identified a novel aPKC phosphorylation site (S1221) within the coiled-coil domain of NuMA. Importantly, the phosphomimetic NuMA-S1221D mutant (NuMA-SD), but not the mutant NuMA-SA phosphomutant, rescued planar spindle orientation upon loss of either aPKC or AGS3. Interestingly, NuMA-SD but not SA restored LGN localization at the cortex early in mitosis. Together, our results indicate a model in which aPKC and AGS3 cooperate through NuMA-S1221 phosphorylation inhibit LGN topromote planar spindle orientation.

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Scribble on the edge: mapping the spatially and structurally resolved interactome of Scribble in health and disease

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The evolutionarily conserved Scribble protein is an important regulator of cell polarity and tumour suppressor in both Drosophila and mammals. Acting as an adaptor protein, Scribble facilitates protein interactions at distinct subcellular localisations, coordinating a range of processes from wound healing and immune response to learning and memory. Scribble achieves this by virtue of its 4 PSD-95/Dlg/ZO-1 (PDZ) domains that account for most of its known interactions, and its spatially restricted basolateral membrane localisation where Scribble interacts with proteins that surround it. Consequently, Scribble’s mislocalisation is implicated in the development of aggressive epithelial cancers and lethal neural tube defects (NTD) in human patients. Despite increasing knowledge on Scribble’s role as a signalling adaptor, the exact mechanism by which its 4 PDZ domains discriminate between its many interactions and exert specificities for certain ligands remains unclear. Furthermore, little is known about how Scribble’s mislocalisation from the plasma membrane to the cytoplasm contributes to its pathogenesis. To investigate Scribble’s role as a spatial organiser of signalling, we have utilised a combination of proteomic, structural and functional approaches in both Drosophila and mammalian models. Using pull-down assays combined with biophysical and X-ray crystallography studies, we have identified evolutionarily conserved PDZ-binding profiles for Scribble-interacting proteins, both known and novel, and have resolved structures of human and Drosophila Scribble PDZ domains bound to various biological partners. To complement this using genetic epistasis studies in Drosophila, we have additionally shown genetic interactions between Scribble and many novel interactors. Finally, using proximity biotinylation and structural analysis, we have now begun to characterise how Scribble’s protein interaction network is altered when mislocalised or mutated in cancer and neural tube defect patients. Taken together, these data begin to provide a mechanistic framework to understand how Scribble functions as an adaptor protein, and how Scribble dysregulation can contribute to disease.
The Endoplasmic Reticulum Scaffolds Cytoplasmic Polarity in The Early C. elegans Embryo

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Cell polarity is a hallmark of most cells and features the asymmetric distribution of factors at the plasma membrane/cell cortex and in the cytoplasm. One way to generate cell polarities is through the local binding of proteins to cellular scaffolds. For example, cortical polarities arise when proteins bind the plasma membrane (scaffold) in one half of the cell. Whether a scaffold also underlies cytoplasmic polarity is not known. We will present our ongoing work showing that the endoplasmic reticulum scaffolds cytoplasmic asymmetries in the one-cell C. elegans embryo. The one-cell C. elegans embryo undergoes an asymmetric cell division during which somatic and germline cell fate factors segregate to the anterior and posterior cytoplasm, respectively. Many germline factors concentrate in germ granules (called P granules in C. elegans), which were previously shown to be in close proximity to mitochondria and the endoplasmic reticulum in ultrastructural studies (Wolf et al, 1983). Consistent with these observations, we find that P granules are associated with the ER through live imaging and that this association persists even after the cytoplasm is extruded from the embryo. Additionally, using optogenetics to stimulate the transport of the ER on microtubules, we observe P granules moving in association with the endoplasmic reticulum. To identify P granule proteins that associate with the ER, 10 endogenously GFP-tagged P granule proteins were analyzed by high-speed spinning disk and the near-TIRF imaging. The Vasa-related protein GLH-1 was unique among these proteins in that it forms dynamic complexes that associate with the ER surrounding P granules. The formation of ER-associated GLH-1 complexes depends on the GLH-1 DEAD-box RNA helicase domain but not on its FG repeat domain. As the embryo polarizes along the anterior/posterior axis, P granules are disassembled in the anterior cytoplasm and assembled in the posterior cytoplasm. The RNA-binding protein MEX-5 stimulates the disassembly of P granules in the anterior cytoplasm. Importantly, we find that MEX-5 associates with the ER in the anterior cytoplasm where it inhibits GLH-1 association with the ER. This inhibition relies on the ability of MEX-5 to bind RNA. Our working model is that the endoplasmic reticulum acts as a cytoplasmic scaffold on which MEX-5 and GLH-1 assemble into asymmetrically distributed RNA/protein complexes, thereby contributing to the establishment of distinct anterior and posterior cytoplasmic domains.

Par polarity proteins in dendritic spine morphogenesis and plasticity

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Neurons are probably the most polarized/compartmentalized cell type in the human body. Their polarity establishment starts with axon/dendrite specification. Further compartmentalization occurs during the formation of dendritic spines, which receive most of the excitatory synaptic inputs in the brain. This raises the exciting possibility that proteins regulating global cell polarization are involved in the localized polarity during dendritic spine morphogenesis and plasticity. Indeed our studies show that the Par (Partitioning-defective) proteins, which are conserved polarity regulators originally identified in C. elegans, play a key role in spine morphogenesis and plasticity. We show that the polarity protein Par1, a Ser/Thr kinase, is involved in dendritic spine morphogenesis downstream of NMDA receptors. Par1
functions through phosphorylating the synaptic scaffolding protein PSD-95 on Ser561. We further found that phosphorylation of this site regulates a conformational switch that is important for bidirectional spine structural plasticity. Using a forebrain-specific Par1c conditional knockout mouse model, we found that Par1c is required for proper spine morphogenesis and cognitive functions \textit{in vivo}. Together, our studies reveal important roles for Par1 in dendritic spine morphogenesis and plasticity, as well as cognitive functions \textit{in vivo}.

\textbf{Fungi}

\textbf{P735}

\textbf{Modeling the rapid emergence of drug resistance in pathogenic fungi}
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Drug resistance has been one of the main causes of treatment failures. It is frequently associated with mutations, which often result in drug target alterations or upregulation of drug efflux. However, resistance seems to emerge at a rate that exceeds what we would predict even from error prone mutation, suggesting that mutation-independent mechanisms could be at play. Using the human fungal pathogen Candida albicans, we discovered an epigenetic mechanism that yields drug resistance at an incredibly high frequency. This drug-adapted state is metastable and lost with passage in the absence of selective pressure. This specific form of drug resistance, which we termed “pararesistance,” is defined as an inducible, heritable, and reversible drug adapted state. We demonstrated that pararesistance does not arise spontaneously in a Luria-Delbrück fluctuation test, a sensitive mutation assay. Since the drug we use is fungistatic, we developed a plate-based colorimetric assay to rapidly distinguish resistant from sensitive colonies. To process our data, we modified the yeast colony classification CellProfiler pipeline to count sensitive and resistant colonies. Through modeling, we found that the Poisson distribution and a mutation rate ranging from $10^{-5}$ and $10^{-4}$ best fit our data, supporting the hypothesis that pararesistance is induced rather than spontaneously acquired. To validate our findings, we are in the process of sequencing multiple pararesistant isolates. If our hypothesis holds true, we expect no shared mutations among pararesistant isolates. Our work provides the first example of an inducible drug-adapted state that is both heritable and reversible, and may represent a bet-hedging strategy that facilitates the rapid emergence of drug resistance.

\textbf{P736}

\textbf{Sexual Parasitism In Fungi: Uniparental Meiotic Progeny From Bisexual Cell-Cell Fusion}
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A fundamental characteristic of eukaryotic organisms is the generation of genetic variation via sexual reproduction. However, some organisms undergo parthenogenesis to produce clonal progeny. Among these parthenogens, some require an opposite-sex partner for initiation but then discard their genome in a process called hybridogenesis exhibiting sexual parasitism. However, this reproductive mode is thus far only known in the animal kingdom. Here, we discovered hybridigenetic reproduction in the fungal kingdom using a human pathogenic basidiomycete, \textit{Cryptococcus neoformans}. \textit{C. neoformans} has two
mating types, \(a\) and \(\alpha\), which fuse to produce a dikaryotic zygote that undergoes hyphal development and complete sexual cycle producing recombinant meiotic progeny. Previously, we employed CRISPR to target centromere-specific retrotransposons leading to the generation of strains with multiple chromosome rearrangements. As a result, these strains showed severe reproductive isolation with the parental wild-type strain. In this study, we show that one of these strains, VYD135, produced delayed, but rare, sporulation with the wild-type strain. Analysis of these progeny showed exclusive uniparental inheritance of nuclear genetic material in the F1 progeny, whereas the mitochondrial genome was always inherited from the \(MATa\) parent, in accord with mitochondrial uniparental inheritance. Whole-genome sequencing revealed the nuclear genome of the progeny was identical with one or the other parental genome, suggesting a complete lack of meiotic recombination between the parental strains. Next, we fluorescently tagged nuclei in these strains and found only one of the two parental nuclei in the sporulating basidia. Tracking of nuclei along hyphae showed loss of one nucleus during dikaryotic hyphal growth, followed by remaining nucleus’s endoreplication and meiosis. Further investigations revealed the occurrence of uniparental sporulation in crosses between natural isolates, in concert with classic biparental sporulation suggesting the presence of such phenomenon in nature. These findings reveal an unusual mode of eukaryotic microbial unisexual reproduction that shares features with hybridogenesis in animals.

P737

**Synthetic control of drug resistance discretizes evolutionary advantages of multicellularity**

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Yeast have attracted the interest of evolutionary biologists by being able to form experimentally evolvable multicellular consortia with increased resistance to environmental stress. Moreover, multicellularity underlies the drug resistance of many pathogenic yeast that cause systemic infections. Despite extensive studies, multicellularity and its role in drug resistance are not yet fully comprehended and controlled experimentally. We pursued the above challenges by studying the genetic bases of clumping multicellularity, its implications in fungal drug resistance, while developing robust computational methods of analyzing and predicting the experimental data obtained in the course of the study. To understand how clumping, a less-studied form of multicellularity affects drug resistance, we sought to abrogate a multicellular clumping phenotype in *S. cerevisiae* Σ1278b and test the drug response of the parental and the mutant strain. Upon deleting the AMN1 gene responsible for mitotic cells separation deficiency and clumping, we obtained a population comprised of single yeast cells, which was more susceptible to common stressors and antifungal agents. Quantitative growth curve analysis (piecewise logarithmic curve fitting) for ancestral and mutant populations in various drug concentrations revealed distinct growth phases that changed in response to drug response concentrations. As opposed to minimal inhibitory concentration (MIC)-based analysis, our growth response assessment is more sensitive to temporal effects of the drugs and can help predict regrowth at the specific dosage. Comparing the growth phase changes revealed the differential effects of stress and antifungals on the clumpy versus unicellular strain. Moreover, the data can inform further computational models of pathogen growth. This work was supported by NIH MIRA grant R35GM122561 and the Laufer Center for Physical & Quantitative Biology.
The rate of whole-genome duplication can be accelerated by hybridization

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Whole genome duplication (WGD) is an important evolutionary force in eukaryotes. While significant progress has been made in the last decade concerning the consequences of WGD, the factors that favour its evolution are less well studied. A large body of work, mostly on plants, showed that hybrid speciation often coincides with WGD. A hypothesis from the early 20th century suggests that parental genetic divergence influences the probability of WGD in hybrids. Bringing two diverged genomes in the same cell would increase the rate of genomic changes and WGD that enable hybrid maintenance on the long term. However, the importance of genetic divergence as a driver for WGD has since been debated. The debate comes from the fact that hybrid species could be more likely to be maintained because WGD increases fitness by restoring fertility and accelerating adaptation, or because hybridization itself increases the rate of WGD. Testing these alternative models requires that natural selection is removed from the equation. Using yeast as an experimental model, we evolved more than a thousand of parallel independent intra- and inter-specific lines of yeast under relaxed selection and measured their spontaneous rate of WGD. We show that WGD is more prone in some lineages, but also can be triggered by hybridization through the combination of some genotypes. We also find that higher WGD rate correlates with higher genomic instability, and that WGD may in turn increase this instability. Together, these results provide evidence that hybridization itself can promote WGD.

Identification of genes influencing production of pulcherrimin, an iron-binding pigment, in the yeast

Kluyveromyces lactis


Pulcherrimin, produced by some yeasts and Bacillus bacteria, is a cyclodipeptide derivative that binds to and immobilizes environmental iron. The consequent iron-scarcity suppresses growth of nearby microbes, which enables the successful use of pulcherrimin-producing yeast as fruit rot biocontrol agents. While the pulcherrimin biosynthetic pathway in bacteria has been known for some time, the distinct putative pathway in yeast has only recently been identified and much about pulcherrimin’s synthesis, ecological role, and regulation remains to be understood. We have taken a genetic approach, studying dysregulated pulcherrimin mutants in the yeast Kluyveromyces lactis to identify new genes and factors that influence pulcherrimin synthesis. One mutated gene is predicted to encode a protein of unknown function, with a conserved C-terminus that is truncated in the mutant protein. The truncation mutant produces pulcherrimin under conditions in which wild type cells do not, and this production responds to changes in carbon source and oxygen level. The increased synthesis of pulcherrimin in this mutant enabled chemical analysis of K. lactis pulcherrimin, confirming that its structure, like that of the
previously characterized Metschnikowia pulcherrima pulcherrimin, was based on a cyclodileucine scaffold. We have recently constructed a heterozygous null mutant in a diploid, which will enable us to determine the null phenotype in a haploid, even if the deletion is lethal. RNA-seq analysis of the truncation mutant revealed differences in expression of the putative pulcherrimin biosynthetic genes and of other genes not yet associated with pulcherrimin production. We are constructing knockout mutants in some of these genes. One of these engineered mutants displayed differences in pulcherrimin production patterns in response to iron and sensitivity to high levels of zinc. Thus, study of dysregulated pulcherrimin mutants has proved to be a successful route to identification of additional genes influencing pulcherrimin production.

Kinesin Biophysics

P740

Cholesterol in cargo membrane amplifies inhibitory effect of MAP tau on kinesin-1
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Motor protein-based transport underlies all eukaryotic cell function and survival; dysfunctions are implicated in many diseases including neurodegeneration. Whereas motor proteins have been extensively studied both in vivo and in vitro, many important questions remain. Chief among these is whether and how the composition of the cargo membrane impacts transport. To address this question, we combined advances in membrane biophysics with established single-molecule optical-trapping assays to characterize the transport of membrane-enclosed cargos in vitro. Our study employed the major microtubule-based kinesin-1 motor and tau, an important microtubule-associated protein (MAP). Tau sterically hinders the binding of individual kinesins to the microtubule and significantly inhibits kinesin-based transport of membrane-free cargos. Remarkably, we found that coupling kinesins via a biomimetic membrane reduces the inhibitory effect of tau, significantly enhancing the transport of membrane-enclosed cargos along tau-decorated microtubules. Further, adding cholesterol to the cargo membrane amplifies the inhibitory effect of tau on kinesin. Combing simulation and modeling approaches, we found that cholesterol hinders the ability of individual motors to search for available binding sites on the microtubule (on-rate), thereby amplifying tau’s steric inhibition of transport. Our study establishes a direct link between cargo-membrane composition and MAP-based regulation of kinesin-1. The combination of experimental and theoretical approaches we developed is generally applicable for interrogating the regulation of motor proteins in a context directly relevant to in vivo scenarios.

P741

Synaptic vesicle precursor transport by UNC-104/KIF1A regulating spatial distribution of synapses: mathematical modeling
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UNC-104/KIF1A transports synaptic vesicles (SVPs) along microtubule tracks in neurons. When not hauling cargo vesicles, UNC-104/KIF1A is assumed to be in an autoinhibited form to reduce the wasteful consumption of ATP\cite{1,2}. This regulatory system controls the efficiency of neuronal transport; the absence of such a system leads to reduced numbers of UNC-104/KIF1A that actively transports SVPs and defects in the generation of vesicles pools and synapses\cite{1}. In order to quantitatively understand how the number of active UNC-104 is related to vesicle pool formation and synapse creation\cite{3}, we observed the distribution and motility of fluorescent-labelled SVPs in \textit{C. elegans} neurons and performed numerical simulations of transport SVPs by UNC-104 based on those observations. \cite{1} S. Niwa et al., Cell Reports \textbf{16}, 2130 (2016). \cite{2} K. Hayashi et al., PCCP \textbf{20}, 3403-3410 (2018). \cite{3} M. P. Klassen et al., Neuron \textbf{66}, 710-723 (2010); Y.E. Wu et al., Neuron \textbf{78}, 994-1011 (2013).

P742

The fast and superprocessive KIF1A predominately resides in a vulnerable one-head-bound state during its chemomechanical cycle
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Kinesin-3 are the fastest and most processive motors of the three neuronal transport kinesin families, yet the sequence of states and rates of kinetic transitions that comprise the chemomechanical cycle are poorly understood. We used stopped-flow fluorescence spectroscopy and single-molecule motility assays to delineate the chemomechanical cycle of the kinesin-3, KIF1A. Our bacterially expressed KIF1A construct, dimerized via a kinesin-1 coiled-coil, exhibits fast velocity and superprocessivity behavior similar to wild-type KIF1A. We established that the KIF1A forward step is triggered by hydrolysis of ATP and not by ATP binding, meaning that KIF1A follows the same chemomechanical cycle as established for kinesin-1 and-2. The ATP-triggered half-site release rate of KIF1A was similar to the stepping rate, indicating that during stepping, rear-head detachment is an order of magnitude faster than in kinesin-1 and kinesin-2. Thus, KIF1A spends the majority of its hydrolysis cycle in a one-head-bound state. Both the ADP off-rate and the ATP on-rate at physiological ATP concentration were fast, eliminating these steps as possible rate limiting transitions. Based on the measured run length and the relatively slow off-rate in ADP, we conclude that attachment of the tethered head is the rate limiting transition in the KIF1A stepping cycle. The fast speed, superprocessivity and load sensitivity of KIF1A can be explained by a fast rear head detachment rate, a rate-limiting step of tethered head attachment that follows ATP hydrolysis, and a relatively strong electrostatic interaction with the microtubule in the weakly-bound post-hydrolysis state.

P743

Kinesin motor number modulates the effect of load on cargo run length
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Kinesin-1 is a major microtubule-based motor that drives long-range transport of cargos in living cells. The distance that kinesin transports its cargos (run length) is strongly hindered by the load the motor experiences. We previously found that load arising from the thermal diffusion of the cargo can significantly shorten run length when the cargo is carried by a single kinesin. It remains uncertain how such quantitative insights into single-molecule effects gleaned \textit{in vitro} can be translated to the \textit{in vivo}
scenario; crucially, cargos in cells are often carried by small teams of motors rather than by a single motor. To close this gap, here we employed Monte Carlo-based simulations to quantify cargo transport by small teams of kinesin-1 under physiological loads. Our simulations utilize experimentally determined single-molecule characteristics of kinesin-1, include loads arising from both cargo diffusion and viscous drag, and encompass a wide range of local viscosities reported for living cells. We model the number of kinesins on the cargo using a Poisson distribution, with the mean motor number approximating the physiological range of 1-2 kinesins per cargo. We used the multi-motor fraction to quantify the mean motor number, because it is an experimentally accessible readout. Strikingly, although groups of kinesin-1 are thought to function non-cooperatively, our simulations indicate that modest increases in kinesin motor number substantially enhance the cargo run length under a variety of load conditions. Our results highlight the potential of kinesin motor number as an important control for cargo transport in vivo.

P744

The Role of Motor Clustering in Kinesin-driven Vesicle Transport

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Kinesin motors drive anterograde transport of membranous organelles along microtubules towards the cell periphery. A single kinesin-1 only travels 1 μm before dissociating from the microtubule, whereas organelles need to travel tens of microns to reach their destinations. Therefore, vesicles usually have multiple kinesin motors and even multiple kinesin isoforms attached, such that when one motor detaches from the microtubule, other motors can sustain the transport. Recent work on the microtubule binding kinetics of kinesin-1 bound to planar lipid bilayers and multi-motor transport characterized by optical traps suggest that kinesin-1 has a slow microtubule on-rate, leading to the prediction that high motor copy numbers are required for long-range vesicle transport. This prediction contrasts with the relatively low motor copy numbers measured on purified intracellular vesicles. To understand this disparity, we reconstituted kinesin-1-driven vesicle transport on synthetic liposomes and explored the possibility that motor organization on the cargo plays a role in regulating transport efficiency. We functionalized POPC liposomes with GFP-labeled kinesin-1 motors through a DNA linker and visualized vesicle transport by TIRF microscopy. Consistent with previous work, transport velocities were insensitive to motor number, but run lengths increased with higher motor copy numbers. By directly measuring GFP fluorescence on the liposomes, we found that significant increases in run length were achieved at relatively low motor copy numbers (< 5). Higher motor numbers also increased the rate of vesicle landing on microtubules. To test the hypothesis that motor clustering enhances vesicle transport efficiency, we used DNA linkers to cluster motors together on the vesicle surface. When vesicles had low motor copy numbers, clustering increased the run length compared to the unclustered case. At high motor numbers that achieved long run lengths on their own, the effect of motor clustering were diminished. Our work showed that motor clustering alone was sufficient to improve cargo travel distance without requiring more motors. This suggests that motor organization on cargo surface may serve as an independent pathway from motor recruitment and motor activation in regulating vesicle transport.
P745

**Cargo binding to microtubules**


Transport of intracellular cargos along microtubules is essential for the function and survival of eukaryotic cells. While we know much about the motors which perform this transport, significantly less is known about how transport is controlled and directed. One step of the transport process which remains little understood is binding of cargos to microtubules. In this poster, we explore what we’ve learned about the binding process through several recent studies. We look at binding in different environments through measurements and simulation both in vitro and in cells. Furthermore, we examine how properties of the cargo (such as diameter and number of motors) affect binding time. Through examining these different cases, we find that cargo rotation plays an important role in determining the time it takes a cargo to bind a nearby microtubule. Using measurements where environment and cargo properties are known, we infer that kinesin motors are likely able to bind faster than previously thought.

P746

**Optical Trapping correlated withIRM, and TIRF microscopy: Studying Cytoskeletal Processes enhanced by Automation**

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Studying the dynamics of cytoskeletal structures and their associated interactions with motor proteins is crucial for understanding the molecular mechanisms governing many essential biological processes such as cell division, cell migration, and mechanosensing. Single-molecule analysis has played a central role in revealing many aspects of these complex and dynamic interactions. Single-molecule techniques provide high spatiotemporal resolution allowing researchers to examine biomolecular interactions one at a time, and at the molecular and cellular level. Such resolution (sub-piconewton in force and a kilo-Hz temporal resolution) is ideal for characterizing subpopulation states and manipulating substrates into unique configurations so that the associated enzymatic activity can be monitored. Here we present a newly developed platform (C-Trap™), which correlates Optical Tweezers with Interference Reflection Microscopy (IRM), and Total Internal Reflection Fluorescence (TIRF) Microscopy. IRM is a recently introduced imaging method that allows visualizing biological structures in 3D without the need of fluorescence labeling and with sensitivity exceeding DIC microscopy. TIRF microscopy provides high resolution fluorescent imaging of specimen near the working surface with high signal to noise ratio resulting in improved single-molecule surface assays. We also demonstrate the faster 2D imaging capabilities obtained – compared to confocal and STED techniques – by introducing widefield microscopy methods to our system. In addition, we demonstrate a user-friendly and high throughput data acquisition method, which is supported by python scripting. The automation of experimental workflows has brought the repeatability and reproducibility of C-Trap™ to the next level. In this work, we will discuss the experimental designs, development of automating experimental workflows and show an overview of the latest results obtained using this single-molecule approach.
Membrane Fission and Coat Proteins

P747

Exchange dynamics of dynamin measured in living cells during endocytic vesicle formation

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During clathrin mediated endocytosis (CME), the GTPase dynamin is recruited to the neck of nascent clathrin-coated vesicles (CCVs) where it oligomerizes into helical filaments. Conformational changes induced by the hydrolysis of GTP catalyze the scission of the vesicle neck. This process has been studied in great detail in vitro but it needs to be established in living cells, where dynamin interactions with SH3 domain containing proteins are critical. Live cell TIRF imaging with the ppH assay (Merrifield et al. Cell 2005; Sposini et al. Nat Protocols 2020) allows the detection of CCV formation with high spatial (~100 nm) and temporal (2 s) resolutions. It has revealed that dynamin is recruited to maturing clathrin coated pits (CCPs) in two phases with a peak at the time of scission (Taylor et al. PloS Biol 2011) but the parameters of its recruitment in living cells remain unclear. To determine these parameters, we have performed live cell imaging of dynamin recruitment at collective and single molecule levels during acute perturbations of its function. First, we showed that Dyngo4a, a cell permeable blocker of dynamin GTPase activity, or GTPγS dialyzed through a patch-clamp pipette, quickly blocked CME and led to the accumulation of dynamin-mCherry at CCPs. Partial block decreased the rate of dynamin recruitment before scission, suggesting that GTPase activity regulates its recruitment. Moreover, photo-activation of DMNPE-GTPγS blocks endocytosis within 4 s while provoking dynamin accumulation on a longer timescale. Second, we showed by FRAP that dynamin exchange is fast and complete and is only moderately impaired by saturating concentrations of Dyngo4a, suggesting that dynamin exchanges with an extra-CCP pool at all times. Finally, we conducted dual ppH/sptPALM imaging in cells expressing dynamin-mEos3.2. Dynamin is recruited to the plasma membrane, diffuses outside of CCPs and is trapped at CCPs. The number of detected molecules increases as scission approaches but single molecules are equally immobilized at all stages of CCP maturation. We conclude that dynamin exchanges with an extra-CCP pool at all times: this would allow for its further recruitment by addition of new binding sites and its ability to narrow the vesicle neck after GTP hydrolysis.

P748

β'-COP functions as a molecular platform to coordinate COPI assembly on membranes

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The essential COPI vesicular coat mediates retrieval of key transmembrane proteins at the Golgi and endosomes following recruitment by the small GTPase, Arf1. ArfGAP proteins regulate COPI coats, but molecular details for COPI recognition by ArfGAP proteins remain elusive. Here we present biochemical and biophysical data revealing how β'-COP propeller domains directly engage the yeast ArfGAP, Glo3,
and quantify the binding affinity. Calorimetry data demonstrate both β'-COP propeller domains are required to bind Glo3 using electrostatic interactions. We identify an acidic patch on β'-COP that interacts with key lysine residues in Glo3. Targeted point mutations in either Glo3 or β'-COP abrogate the interaction in vitro. We demonstrate how loss of the β'-COP/Glo3 interaction drives Ste2 mis-sorting to the vacuole and aberrant Golgi morphology in budding yeast. We present a model for a β'-COP/Glo3/Arf1 complex on membranes by combining biochemical data with an X-ray crystal structure of the Glo3 GAP domain. Together, these data suggest cells require the β'-COP/Glo3 interaction for cargo recycling via endosomes and the TGN, where β'-COP serves as a molecular platform to coordinate binding to multiple protein partners, including Glo3, Arf1, and the F-subcomplex.

P749

**Induced Nanoscale Membrane Curvature Nucleates Endocytic Sites, Reduces Endocytic Lifetime, and Rescues Clathrin Knockdown**

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Clathrin-mediated endocytosis (CME), the main route of receptor uptake in eukaryotes, requires rapid membrane remodeling on the scale of 50 nanometers to produce spherical vesicles from flat membrane. We used a combination of electron beam lithography and UV-nanoimprint lithography to create a set of glass-like nanoscale substrates, suitable for light microscopy techniques, with curved features of diameters ranging from 50-1000 nanometers to mimic the stages of CME progression. We imaged genome-edited, fluorescently tagged proteins that function at distinct CME stages in MDA cells. We found that proteins from all CME stages accumulate at sites of high membrane curvature, with three- to four-fold enrichment over flat membrane, and that the lifetimes of early endocytic proteins AP2 and clathrin are shortened significantly as curvature increases. We found that the protein Epsin1, which binds directly to PIP2 and bends the inner leaflet in endocytosis, strongly localizes to sites of high curvature but does not turn over for the duration of our imaging. Recruitment of the GTPase dynamin2, responsible for membrane scission, peaks significantly earlier at sites of high curvature. Together, these data suggest that generation of curvature may be rate-limiting in CME. To test whether induced curvature can rescue a defect in endocytosis, we knocked down clathrin and AP2 using siRNA. Curvature rescued AP2 recruitment in clathrin knockdown cells, as well as recruitment and turnover of dynamin2 in clathrin knockdown cells and in cells treated with the CME inhibitor pitstop2. However, upon knockdown of AP2, clathrin and dynamin2 are not recruited to sites of induced membrane curvature. To test whether exogenous curvature rescues productive endocytosis from knockdowns, we performed transferrin uptake assays and found that induced curvature partially rescues transferrin uptake upon knockdown of clathrin, but not AP2. These data are consistent with clathrin acting as a Brownian ratchet, locking in the energetically costly membrane curvature as CME progresses, and that the presence of induced curvature may bypass this step, speeding up the production of vesicles.

P750

**The Structure and Function of the Commander Membrane Trafficking Machinery**

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Membrane compartmentalisation is a defining feature of all eukaryotic cells and we have evolved sophisticated protein machineries to control the flow of transmembrane molecules and lipids between organelles. One of the most dynamic membrane bound compartments is the early endosome, which controls the sorting of transmembrane cargos as they transit through the secretory and endocytic system. The Collins lab is deciphering the molecular mechanisms that control this membrane sorting using a combination of structural and cellular biology. ‘Retromer’ is one of the best characterised endosomal transport protein machines, and is mutated in Parkinson’s Disease, and recently we and others identified a ‘Retromer-like’ protein complex named ‘Retriever’. Retriever forms a large macromolecular assembly with other proteins including the so-called COMMD and CCDC complexes and a member of the sorting nexin protein family called SNX17. The mega-complex has been dubbed ‘Commander’ and plays an important role in endosomal trafficking of many receptors including integrins, lipoprotein receptors, ATP7A copper transporters, ENaC epithelial sodium channels, and amyloid precursor protein and other receptors. Here I will present a number of structures of both individual components of the Commander complex as well as higher order assemblies. This in combination with a number of biochemical and biophysical techniques has given us insight into how Commander is assembled, interacts with transmembrane cargos, and regulates their recycling or degradation.

P751

Endocytosis against high turgor pressure is made easier by partial protein coating and a freely rotating base

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During clathrin-mediated endocytosis, a patch of flat plasma membrane is deformed into a vesicle. In walled cells, such as plants and fungi, the turgor pressure is high and pushes the membrane against the cell wall, thus hindering membrane internalization. Using mathematical modeling, we studied how a patch of membrane is deformed against turgor pressure by a point force. We found that a large amount of force is needed to merely start deforming the membrane and an even larger force is needed to pull a membrane tube. The magnitude of these forces strongly depends on how the base of the membrane is constrained and how the membrane is coated with curvature-generating proteins. In particular, these forces can be reduced by partially but not fully coating the membrane patch with curvature-generating proteins. Our theoretical results show excellent agreement with experimental data.

P752

Determination of geometric features that distinguishes the constant area model from the constant curvature model for clathrin-mediated endocytosis

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During clathrin-mediated endocytosis, a patch of flat plasma membrane is invaginated to form a vesicle. How the clathrin coat deforms the membrane remains unclear and two main hypotheses have been
debated. The “constant area” hypothesis assumes that clathrin molecules initially form a flat lattice which is then rearranged into a spherical coat that deforms the membrane by changing its intrinsic curvature while keeping the coating area constant. The alternative “constant curvature” hypothesis assumes that the intrinsic curvature of the clathrin lattice remains constant during the formation of a vesicle while the surface area it covers increases. We used mathematical modeling to test these two hypotheses under conditions relevant to mammalian cells or to yeast and plant cells. We found that the curvature at the tip of the endocytic pit, which can be directly measured from electron micrographs, can be used to distinguish the two hypotheses. We compared our theory with experimental data from mammalian cells and found an excellent fit only under the constant area hypothesis. For yeast cells, our model shows that both hypotheses can produce vesicles but neither of them produces shape similar to the experimental data. Our model demonstrates that a pulling force generated by actin polymerization is required to produce elongated shapes seen in yeast cells but this force prevents the membrane from being pinched off into a vesicle. These results predict that an additional mechanism is required to complete membrane fission in yeast.

P753

Investigating the Structure and Function of the Arf GEFs Gea1 and Gea2
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In eukaryotes, vesicle formation at the Golgi complex is initiated by the conserved small GTPase Arf1. In budding yeast, Arf1 localization and activity is controlled by three separate guanine nucleotide exchange factors (GEFs): Gea1, Gea2, and Sec7. The paralogues Gea1 and Gea2 are responsible for activating Arf1 at the early Golgi to commence COPI vesicle formation which is essential for retention of resident proteins at the endoplasmic reticulum and early Golgi. However, the mechanism by which Gea1 and Gea2 are recruited to the Golgi at the right place and time to activate COPI vesicle formation is unclear. Having a structure of the full-length protein would facilitate these efforts, however past attempts at solving the crystal structure have failed. I have taken a different approach by using CryoEM to further understand these proteins and their unique domain structures. I will present my progress in generating a preliminary low-resolution map of Gea2 bound Arf1. I will also discuss technical and computational challenges I have faced and new questions the data has unveiled.

P754

Reconstituting clathrin-mediated vesicle formation at mitochondria
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Clathrin-mediated vesicle formation is a major membrane remodelling event during membrane traffic in eukaryotic cells. Clathrin-coated vesicles (CCVs) are formed at the trans-Golgi network and early/recycling endosomes during intracellular traffic, and at the plasma membrane (PM) during endocytosis. Besides cargo, adaptors and clathrin, there are other factors involved: accessory proteins, phospholipid composition and curvature of the membrane. While these factors have all been shown to contribute to the formation of CCVs; their specific roles are not very well understood. Here, we asked what are the minimal requirements to form a CCV? To answer this question, we reconstituted CCV
formation at an intracellular membrane: the mitochondrial outer membrane (MOM). We used the clathrin binding domains of the β2 subunit of the AP2 complex, as a ‘hook’ which can be attached inducibly to an ‘anchor’ protein on a membrane surface. We found that rerouting the hook to an anchor at the MOM is sufficient to form CCVs. Electron microscopy and live cell imaging reveal that all steps of clathrin mediated vesicle formation from initiation to maturation and scission were reconstituted on mitochondria. Clathrin-coated pits on the mitochondria (termed MitoPits), form within minutes after induction. Mitopits are double membraned invaginations of around 100 nm diameter with a clear clathrin coat. This observation suggests enough force is generated by our synthetic system to deform both the inner and outer mitochondrial membrane. We observed scission of MitoPits in real time and the identity of the scission molecule responsible was investigated. Strikingly, Mitopits tend to form on mitochondrial surfaces that have higher curvature. Given the differences in lipid composition of mitochondria and other cellular membranes, our system suggests that phospholipid composition is not a key factor for clathrin-mediated vesicle formation. The formation of Mitopits also calls into question the importance of accessory proteins in clathrin-coated pit formation. In summary, our work identifies the minimal factors required for clathrin-mediated vesicle formation.

P755

Evidence for Involvement of the Eps15 Homology Domain Protein, EHD4, in Endosomal Fission and EHD1 Recruitment

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Objective

Determine the role of EHD4 in the process of endosomal fission and recruitment of the well-characterized paralog EHD1 to sorting endosomes (SE). Background

Upon internalization, receptors are trafficked to SE where they are organized into budding vesicles that undergo fission and transport within the cell. Eps15 Homology Domain Protein 1 (EHD1) belongs to a family of four mammalian paralogs and has been implicated in catalyzing the fission process releasing such endosome-derived vesicles for transport. The fourth EHD protein, EHD4, is the least characterized EHD protein. EHD1 shares a high percentage of homology with the third EHD protein, EHD3, and the two have been shown to heterodimerize. EHD1 and EHD4 exhibit a similar percentage of homology, although the nature of their interaction and its impact on endosomal fission are not known. Hypothesis

Based on its localization to endosomes and its homology to EHD1 (which has already been implicated in endosomal fission), we hypothesized that EHD4 is involved in the process of vesicle fission from endosomes through its heterodimerization with EHD1. In addition, we theorized that EHD4 interacts with endosomal proteins such as Syndapin2 and Rabenosyn-5 to facilitate recruitment of EHD1/EHD4 heterodimers to SE. Methods

We tested whether endosome fission is impaired in CRISPR/Cas9 EHD4-null cells, as well as in cells treated with EHD4-shRNA or EHD4-siRNA. We also utilized CRISPR/Cas9 EHD1-null cells and CRISPR/Cas9-EHD1- and EHD4-null cells to observe the effects of EHD1, EHD4, and EHD1/EHD4 loss on endosomal fission. Experiments involving the depletion of Rabenosyn-5, Syndapin2, and MICAL-L1 respectively in CRISPR/Cas9 EHD1-GFP cells were used to identify which proteins are required for EHD1/EHD4 heterodimer localization to endosomes and to facilitate endosomal fission. Results/Data

We have demonstrated that EHD4 depletion by shRNA or siRNA leads to enlarged endosomes in shRNA and siRNA (as measured by endosome diameter) and accumulation of select internalized receptors within these endosomes. In addition, loss of EHD4, Syndapin2, MICAL-L1, and Rabenosyn-5 (all of which are EHD1
endosomal interaction partners) led to decreased recruitment of EHD1 to endosomes and enlarged SE structures. **Conclusions** Overall, our data point to a likely role for EHD4 as a key interaction partner and recruiter of EHD1 to the endosomal membrane and its involvement in the process of endosomal fission, thus identifying EHD4 as an essential component of the EHD1-fission machinery at SE.

**P756**

**Role for class II PI3K in T-tubule Remodelling**

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Specialized membrane compartments called T-tubules in large, highly differentiated myofibers are essential to support muscle contraction and integrity. Transversal tubule (T-tubule) allow for synchronized myofibril contraction. Very little is known about how the T-tubule membrane network is formed and maintained. Significance of T-tubule network is mostly known from their link to myopathies. To address the challenges of studies in differentiated muscles, we are leveraging the genetic and live imaging advantages of studying T-tubule requirements in Drosophila body wall muscles. Our lab found that a regulated function for Dynamin large GTPase, initiates T-tubule disassembly necessary for a wildtype developmental muscle remodeling program, which may explain dynamin overexpression resulting in T-tubule fragmentation and the association of dominant mutations in the conserved mammalian dynamin, DNM2, with myopathy. In addition, dynamin has a well-described function in endocytic vesicle formation. We asked whether regulation of dynamin in T-tubule remodeling shares requirements with regulation of dynamin in endocytosis. Specifically, the late steps in clathrin-mediated endocytosis are directed by Class II PI3-kinase (PI3KC2A), which generates a local PI(3,4)P2 pool that recruits SNX9 and subsequently dynamin for membrane scission. Flies encode for a single PI3KC2 homolog (Pi3K68D) previously implicated by our lab in developmental remodeling of abdominal muscles. We found that PI3KC2 - like dynamin - is required at initiation of muscle remodeling for T-tubule disassembly. Interestingly, two distinct PI3KC2 splice variants are both required for the process. Disruption of PI3KC2 function also blocked inappropriate T-tubule disassembly induced by dynamin overexpression, raising the possible significance of mammalian PI3KC2A in DNM2 dominant disease conditions. Our initial studies show that the single Drosophila SNX9/SNX18 homolog (SH3PX1) is also required for T-tubule disassembly. Together, our results suggest that there are shared mechanisms for regulation of dynamin function in endocytosis and T-tubule disassembly. Current studies are exploring a model for phosphoinositide dependence and site of dynamin function at T-tubule membranes in regulation of disassembly.
Microtubule-Associated Proteins

P757

Doublecortin domains have evolved into distinct subtypes in metazoans

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The evolution of the microtubule cytoskeleton has included an expansion in the number of tubulin genes and a diversification of microtubule-associated proteins (MAPs). The Doublecortin family is a group of MAPs containing microtubule binding motifs called DC domains, which evolved from a primitive β-grasp fold shared with ubiquitin. The DC family is best known for its founding member, human DCX, which causes hereditary brain disorders when mutated. Despite their association with the nervous system, DC family proteins are present in eukaryotes from unicellular alveolates to mammals. The phylogeny of the DC family was last analyzed in 2006, so we reanalyzed the family in light of new family members, additional clinical data, and our significantly expanded biophysical view of DC family proteins in order to obtain a unified view of the evolution of the DC family. We wondered when DC domain proteins first arose and whether the tandem domain structure is universal. We found DC proteins with only a single DC domain in early unicellular eukaryotes, suggesting that the tandem domain structure arose later. Notably, across all organisms, the two DC domains, which we call DC1 and DC2, had unique signatures that separate them in sequence alignments. Our results suggest a duplication event in a very early eukaryote followed by rapid divergence. Additionally, our phylogeny produced two subtypes of DC1 domain. One subtype, which we call DC1+, which is found in DCX, contains an extra loop of 4 amino acids, as observed in recent cryo-EM structures. The differences between DC1 and DC2 explain why the 2 domains bind preferentially to different conformations of the microtubule binding site. DC domains bind to microtubules at the vertex of 4 tubulin dimers, and thus contact the lattice at 4 contact sites. Surprisingly, the microtubule binding residues are mostly conserved among mammals, arthropods, nematodes and alveolates, with three of four similar contact sites. The divergence at the 4th contact site, along with overall shifts in the protein’s fold, may allow the DC domains to bind to specific tubulin conformations and/or adapt to divergent microtubule lattices. We conclude that DC family proteins cannot be regarded as simple “tandems”. Rather, the phylogeny suggests that distinct subtypes of DC domains were selected for early in the evolution of the DC family.

P758

Circulating white blood cells exhibit low expression of kinesin genes in public RNA sequencing datasets

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Microtubules (MTs) are cytoskeletal polymers that participate in processes common to all cells, such as division and intracellular tracking, and in cell type-specific biology, such as flagellar motility. Investigations of cell type-specific MT biology usually start by studying the unique properties of a cell’s MT architecture, an approach which may overlook specialized functions of MTs and MT-associated proteins that are not observable with electron microscopy. As such, we sought to profile MT biology across various tissues using an alternative approach based on public gene expression data. We first generated an inclusive list of genes involved in MT biology that we call the “microtubulome.” We then
analyzed mRNA expression of these genes in the Tissue Atlas RNA sequencing dataset, which covers 62 tissues and cell types. Hierarchical co-clustering revealed that circulating white blood cells have low expression of most kinesin (KIF) genes and several tubulin genes compared to cells in other tissues, suggesting lower reliance on MTs and MT proteins for cell organization. Additionally, differential analysis of the RNA sequencing dataset from the Cancer Cell Line Encyclopedia found that KIF21B is an exception, with higher expression in cancer cells of hematological and lymphoid origin compared to non-hematological and lymphoid cells. All other kinesins were less expressed. This suggests that KIF21B may have a function that is especially important in white blood cells. We plan to extend our analysis of microtubulome transcriptional regulation in single cell RNA sequencing data, which will allow us to compare the MT biology of diverse cell types within tissues. Overall, our computational approach to studying the microtubulome is uncovering common and diverse MT biology in different cell types in a manner that complements conventional cell biological assays.

P759

Medium throughput cell-free approach to study Microtubule-Associated Proteins using TIRF-reconstitution assays

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The Microtubule (MT) cytoskeleton is complex dynamic filaments involved in a plethora of functions such as cell division, cell shape, ciliary beating, neuronal differentiation. Strict regulation of microtubule functions is therefore of high importance for the cellular homeostasis, and any perturbations could potentially lead to diseases like cancer, ciliopathies and neurodegeneration. At the protein level, there are accumulating studies showing that MT properties can be controlled via interaction with a large variety of microtubule-associated proteins (MAPs). Our knowledge of MAPs has been enriched over time, but up to this day no systematic studies exist that aim to describe and categorize these proteins according to their binding mechanisms and structural effects on microtubules. In the present work, we have developed an assay for rapid and systematic analysis of around 50 MAPs, using cleared lysates of cultured human cells in which we overexpress the MAPs of interest. The dynamic behaviour of growing MT in the presence of different MAPs were imaged using TIRF microscopy. This allows us to study the behaviour of many MAPs in a situation close to their natural environment, but eliminating complexity coming from different organelles and crammed cytoskeleton filaments inside the confined intracellular space. Indeed, most MAPs were nicely soluble in our extract approach, while purification often led to protein precipitation. Our novel approach allowed us to define several novel proteins as bona-fide MAPs. We show that previously uncharacterized MAPs have strikingly different effects on MT polymerization and MT structure, thus creating a variety of distinct MT arrays. Also, we extended this cell-free pipeline to study MAP-MT structures by cryo-electron microscopy, to study dynamic MT carrying patient mutations, used to produce MAPs to test its sensitivity against tubulin PTMs and to study the role of MAPs in actin-MT crosstalk. Our approach would allow for a better mechanistic understanding of how MAPs and MTs together control cytoskeleton functions.
Tau forms oligomeric complexes on microtubules that are distinct from pathological oligomers in disease

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Tau is a microtubule-associated protein, which facilitates the assembly and stability of neuronal microtubules in humans. Accumulation of tau into insoluble aggregates known as neurofibrillary tangles (NFTs) is a pathological hallmark of several neurodegenerative diseases. The current hypothesis is that small, soluble oligomeric tau species preceding NFT formation could be causing loss of tau function and toxicity. Here, using single molecule localization microscopy (SMLM), we show that, in vivo, tau forms small oligomers on microtubules under physiological conditions. These physiological oligomers are distinct from pathological oligomers and could be pre-cursors of aggregation in pathology. Further, using SMLM and an unsupervised shape classification algorithm that we developed, we show that different tau phosphorylation states are associated with distinct higher order aggregate species in pathology. Our work elucidates tau’s nanoscale composition under physiological and pathological conditions in vivo.

UNC-45A is novel ATP-independent MT severing protein overexpressed in cancer and neurodegenerative diseases

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For the longest time, cancer and neurodegenerative diseases have been considered at the opposite end of the spectrum; cancer is characterized by dividing cells that resist death, neurodegeneration is associated with death of post-mitotic cells. Today genetic and cell biology studies show common pathways in cancer and neurodegeneration including proteins that regulate microtubule (MT) stability. Tau for instance, a MT-associated-protein (MAP) largely expressed in brain and whose hyperphosphorylation is associated with Alzheimer’s disease (AD), is abnormally expressed in human cancers. Furthermore, drugs targeting MT stability is an established anti-cancer approach and stabilization of neuronal MTs can attenuate neurodegenerative diseases. We are one of the few laboratories in the world to study the roles of UNC-45A, a highly conserved member of the UCS (UNC-45/CRO1/She4p) protein family, in mammalian cells. We (and others) have shown that UNC-45A: is required for cytokinesis and is overexpressed in a number of human cancers (Bazzaro et al., 2007; Guo et al., 2011), plays a role in exocytosis in immune cells (Iizuka et al., 2015), and is required for axonal growth (Iizuka et al., 2017) via regulation of acto-myosin contractility. While UNC-45A is implicated in regulating the actomyosin system (Barral et al., 2002; Lehtimaki et al., 2017), it is becoming clear that it has additional functions including regulation of centrosomal positioning (Jilani et al., 2015). Importantly, we have recently shown that UNC-45A is a novel Microtubule-Associated Protein (MAP) with MT depolymerizing properties in normal and cancer cells (Habicht et al., 2019; Mooneyham et al., 2019), and that UNC-45A acts on MTs independent of its myosin II binding domain and in presence of the...
myosin II inhibitor blebbistatin (bioRxiv 2020.06.20.163048; doi: https://doi.org/10.1101/2020.06.20.163048). Here we present our new, unpublished data that UNC-45A destabilizes MTs by weakening the MT lattice in an ATP-independent manner. This mechanism is distinct from the ones of other known MT lattice destabilizing proteins including katanin. We also for the first time show that UNC-45A is largely expressed in human brain where it co-localizes with MTs with a predominant localization to the proximal portion of the axonal shaft, the main site of neuronal MTs severing. Furthermore, we show that in neurons UNC-45A overexpression leads to MTs bending and breaking and to loss of MT mass. Lastly, using a large cohort of human clinical specimens of healthy brains, and of individuals with Mild Cognitive Impairment (MCI) and AD, we show that UNC-45A is overexpressed in MCI and AD and associated to a loss of MT mass. The implication is that UNC-45A could be a biomarker and a therapeutic target for Alzheimer’s disease.

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**Clasp2 mediates co-organization of microtubules and actin**

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Coordination between the microtubule and actin cytoskeletons is essential in cell motility, neuronal growth cone guidance, and wound healing. In a cellular context, this coordination is controlled by crosslinking proteins and when disrupted can lead to disease. While studies in cells have identified important crosslinking proteins, the mechanisms underlying the interactions between microtubules and actin filaments remain largely unknown. Members of the CLASP (Cytoplasmic Linker-Associated Protein) family of proteins have been implicated in the cytoskeletal crosstalk. However, the specific dynamic interactions between microtubules and actin mediated by CLASPs are less understood. Here, we demonstrate the direct interaction of CLASP2α with actin using biochemical pull-downs with purified protein components. We then investigate CLASP2α’s colocalization with microtubules and actin using an *in vitro* reconstitution assay, visualized by total internal reflection fluorescence microscopy (TIRFM). Our results demonstrate that CLASP2α directly interacts with actin *in vitro*, preferentially colocalizes with bundled F-actin, and is able to increase the coalignment of microtubules along F-actin bundles. Furthermore, we characterize interactions between dynamic microtubules and bundled F-actin over a range of CLASP2α concentrations, demonstrating that CLASP2α increases the frequency of microtubule zippering events along bundled F-actin. Taken together, our results elucidate the direct biochemical mechanisms underlying microtubule-actin coordination by CLASP2, essential in a number of cellular processes.

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**Tissue-specific expression profile of a plant-specific microtubule binding protein family, MACET**

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With nearly 7 billion people on earth, a question that arises is: how can we keep up with dwindling renewable resources? One important resource is biomass in the form of wood. The current need for lumber exceeds new growth, especially in underdeveloped nations, which leads to deforestation and global climate shifts. Wood is composed of specialized vascular cells that conduct water and nutrients called Xylem. Xylem cell walls are reinforced with lignin which provides structural support. The thick,
Lignified cell wall means land plants adapted a unique mechanism for cell division and growth. In order for plant cells to divide, they create a partition between daughter cells during cytokinesis, called the cell plate, which matures into the cell wall. Cell plate material is trafficked to the division site via a microtubule structure called the phragmoplast. The phragmoplast is made of two interdigitating rings of microtubules which expand from cell center to meet the mother cell wall as the cell plate is deposited. The phragmoplast, unlike structurally stable midbody in animal cytokinesis, is dynamic. As the phragmoplast is specific to plants, our research addresses plant-specific proteins that regulate microtubule activity in the phragmoplast. To this end, we have identified a family of plant-specific microtubule binding proteins in the phragmoplast named MACET. My research focuses on characterizing the spatial and temporal expression of MACET family protein activity. In Arabidopsis plants, we can ligate the native promoter of each MACET gene to the sequence of the enzyme, β-Glucuronidase. A special substrate of β-Glucuronidase, X-gluc, will stain plant tissue blue when cleaved by β-Glucuronidase as it is driven by the native promoter. This reporter system shows that MACET promoters are active in meristematic tissue, differentiating cells and the vascular system. By further studying where this protein is active in plant cells, we can determine the role of MACET in plant cell division. MACET function will add to our understanding of how phragmoplast microtubule dynamicity remains ordered during cytokinesis. By further understanding the function of MACET protein in the phragmoplast, we can model how to genetically engineer plant cells to increase rates of cell division and therefore increase biomass.

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**Fyn-mediated phosphorylation of tau differentially regulates the transport of early endosomes and lysosomes**

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Different sets of kinesin and dynein motors associate with and transport specific organelles such as early endosomes and lysosomes. Microtubule-associated proteins (MAPs) regulate transport in a motor-specific manner. MAP tau strongly inhibits kinesin-1 and kinesin-3 processivity, while kinesin-2 and dynein are less sensitive to tau. Phosphorylation tunes the binding kinetics of tau to microtubules. Over 85 putative phosphorylation sites have been identified on tau, phosphorylated by multiple kinases including GSK3β, AKT, MAPK, and Fyn. Fyn phosphorylates tau at tyrosine 18, resulting in decreased affinity to microtubules and weakened inhibition of kinesin-1. We examined the effects of tau and fyn-mediated tau phosphorylation on microtubule organization and the transport of early endosomes and lysosomes in COS-7 cells. Phosphorylation relieves the inhibition of lysosomal motility by tau. Juxtanuclear lysosomes are not inhibited by phosphomimetic tau (Y18E), whereas peripheral lysosomes are inhibited by both WT (wild-type) and phosphomimetic tau. In contrast, early endosomal motility is inhibited by both tau Y18E and WT tau. Overall, early endosomes are more sensitive to the presence of tau compared to lysosomes, which are known to associate with different sets of kinesin motor proteins. We expect that the types of engaged kinesins govern the disparate responses of different types of cargoes to tau. Motility assays on reconstituted microtubules show that phosphomimetic tau strongly inhibits kinesin-3, in contrast to kinesin-1 motility, which is partially rescued by tau phosphorylation at
Y18. Cargoes positioned in central and peripheral regions of the cell exhibit differential motility in the presence of tau, likely due to different motors driving their transport. Together, our results show that tau and its phosphorylation specifically regulate the motility of differing organelles through motor-specific regulation of kinesins on the microtubule lattice and modulating microtubule network organization.

mTOR and MAP Kinase Signaling

P765

The role of intestinal TOR signaling in metabolic responses to bacterial infection.
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Animals in their natural ecology are often exposed to environmental stressors (e.g. starvation, extreme temperature, hypoxia, pathogens) that can affect their physiology, development and lifespan. An important question in biology is how animals sense these stresses and, in response, adapt their metabolism to maintain homeostasis and survival. In some cases, specific tissues function as stress sensors to control whole body adaptive responses. One well-studied example is the Drosophila intestine. In response to oral pathogenic bacterial infection, the fly gut controls both local and systemic antibacterial immune responses. Recent work shows that the gut also controls whole-body metabolic changes to promote infection tolerance. Here, we show that one way that the fly gut mediates these adaptive metabolic responses is via induction of target-of-rapamycin (TOR) kinase signaling. TOR is a well-established regulator of metabolism that has classically been shown to be activated by growth cues and suppressed by stress conditions. Interestingly however, we found a rapid increase in TOR activity in the fly gut in response to bacterial infection stress. Furthermore, we showed that blocking this TOR induction reduced survival upon infection, whereas when we genetically enhanced TOR signaling specifically in the intestine, we saw extended lifespan. Our data suggest that these protective effects of gut TOR signaling on organismal survival may be mediated through altered whole-body lipid metabolism. We found that bacterial infection increased expression of lipases, and induced both intestinal and whole-body depletion of lipid stores. In contrast we found that genetic activation of TOR in the intestine induced expression of transcription factors and enzymes that promote lipid biogenesis, and led to increased whole body lipid stores. Moreover, we found that TOR was required for upregulation of several gut-derived signaling peptides that have been shown to communicate with the brain and fat to control lipid metabolism. Our data supports a model in which induction of TOR signaling in the intestine represents a host adaptive response to counteract infection mediated loss of whole-body lipid stores in order to promote survival.
mTORC1 deregulation and increased invasiveness cohere with dispersed endolysosomes in high-grade bladder cancer

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Late endosomes/lysosomes (endolysosomes) emerge as a potential regulatory hub during cancer. Here, we investigate the intracellular landscape of this organelle in a collection of bladder cancer cell lines and normal human urothelium cells under standardized culture conditions. We find that high-grade bladder cancer cells are characterized by scattered endolysosomes that are accompanied by an altered cellular pH homeostasis and major changes of mTORC1 regulation. Mechanistically, we reveal that mTORC1 substrate specificity is altered, and mTORC1 responsiveness to endolysosome positioning is lost in high-grade cancer cells compared to low-grade cells, highlighting unexpected mechanisms of mTORC1 deregulation in the bladder cancer model. Because endolysosome positioning was critical for invasion from 3D spheroids, our results indicate that changes in their cellular positioning and ability to support signaling, strongly impact cancer cell behavior. Thus, monitoring detailed changes of endolysosomes at different steps of cancer disease reveals intricate spatial and temporal dimensions of tumorigenesis. Our study reveals significant changes of endolysosomes in bladder cancer progression, highlighting endolysosome dysfunction as a fundamental driving progress in malignancies. The identified alterations in endolysosome positioning and associated mTORC1 signaling regulation could help to stratify emerging therapeutic strategies targeting the endolysosomal compartment.

P767

Maladaptive nutrient signalling sustains the m.3243A>G mtDNA mutation

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Mutations of the mitochondrial genome (mtDNA) cause a range of profoundly debilitating clinical conditions for which treatment options are very limited. Most mtDNA diseases show heteroplasmy - tissues express both wild-type and mutant mtDNA. While the level of heteroplasmy broadly correlates with disease severity, the relationships between specific mtDNA mutations, heteroplasmy, disease phenotype and severity are not well-understood. Among human mtDNA mutations, the m.3243A>G mutation is the most prevalent one of mtDNA diseases and the major cause of mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes syndrome (MELAS). Using live-cell imaging, we found that mitochondrial dysfunction caused by the m.3243A>G mutation increases glucose dependence in the mutant cells, resulting in redox imbalance and oxidative stress. Metabolomics further revealed that the m.3243A>G mutation remodels glucose and lipid metabolism towards increased anabolic biosynthesis and lipid accumulation. Moreover, RNA-sequencing and immunofluorescence showed that the m.3243A>G mutation leads to metabolic changes, promoting the upregulation of the PI3K-Akt-mTORC1 axis in patient-derived cells and tissues. Remarkably, pharmacological inhibition of PI3K, Akt, or mTORC1 for 6-12 weeks activated mitophagy, reduced mtDNA mutant load and rescued
cellular bioenergetics. We further established that the reduction of the mutant load is cell-autonomous by long-term cell growth/death analysis and single-cell PCR. In addition, the reduction was prevented by inhibition of mitophagy, showing that mitophagy is necessary for the rescue. Of note, we also examined the effects of PI3K-Akt-mTORC1 inhibition in cells carrying the m.8993T>G mtDNA point mutation (a gift from Dr Minczuk, MRC MBU), in which inhibition of the axis had no impact on mutant load. This suggests that hyperactivation of the PI3K-Akt-mTORC1 axis is relatively disease-specific and perhaps points to mechanisms that define different disease phenotypes between mitochondrial diseases, and suggesting that therapeutic options should be considered separately for each disease related to mtDNA mutations. Together, these data strongly argue that the chronic activation of the PI3K-Akt-mTORC1 axis, presumably as an adaptive response to impaired oxidative metabolism, instead serves as a maladaptive response in the m.3243A>G mutation disease model. Thus, the PI3K-Akt-mTORC1 axis represents a therapeutic target with translational potential that may benefit people suffering from the consequences of the m.3243A>G mutation.

P768

Dysfunction of Lip1 causes hyperphosphorylation of ceramide synthase Lag1 dependent on TOR complex2- and Pkh1/2-Ypk1 signaling pathways in budding yeast
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Saccharomyces cerevisiae adapts to environmental stresses such as high-temperature stress by precisely controlling the amount of sphingolipids in the plasma membrane. However, the details of the regulatory mechanism of sphingolipids metabolism have not yet been elucidated. In this study, we focused on S. cerevisiae LIP1 to elucidate the regulatory mechanism of the synthesis of ceramide, which is a precursor of sphingolipids. S. cerevisiae LIP1 encodes the regulatory subunit that forms a complex with the ceramide synthase catalytic subunits, Lag1/Lac1, localized on the endoplasmic reticulum membrane. To understand how Lip1 regulates the function of Lag1/Lac1, we generated strains in which chromosomal LIP1 promoter was replaced with a Tet-off promoter capable of regulating the expression in a doxycycline (Dox)-dependent manner. The lip1-1 strain, one of the strains obtained by the promoter substitution exhibits severe growth inhibition and remarkable sphingolipid synthesis abnormality in the presence of Dox. Using lip1-1 strain, we investigated the effect of reduction of ceramide synthesis on TOR complex 2 (TORC2)-Ypk1 signaling, which senses the complex sphingolipid level at the plasma membrane and promotes sphingolipid synthesis. In lip1-1 cells in the presence of Dox, Ypk1 is activated by both upstream kinases, TORC2 and yeast PDK1 homologues, Pkh1/2, which resulted in hyperphosphorylation of Lag1 known as one of Ypk1-substrates. Furthermore, the hypomorphic allele of TORC2- and Pkh1/2-singnalings are indispensable for the promotion of ceramide synthesis in yeast.
Cryptosporidial Infection Suppresses Intestinal Epithelial Cell MAPK Signaling for Evasion of Host Antimicrobial Defense

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*Cryptosporidium* is a genus of protozoan parasites that infect the gastrointestinal epithelium of a variety of vertebrate hosts. Intestinal epithelial cells provide the first line of defense and play a critical role in the initiation, regulation, and resolution of host immunity against *Cryptosporidium* infection. To counteract host defense response, *Cryptosporidium* has developed strategies of immune evasion to promote parasitic replication and survival within epithelial cells but the underlying mechanisms are largely unclear. Using various models of intestinal cryptosporidiosis, we found that *Cryptosporidium* infection caused suppression of the mitogen-activated protein kinase (MAPK) signaling in infected murine intestinal epithelial cells. Whereas expression levels of most genes encoding the key components of the MAPK signaling pathway were not changed in infected intestinal epithelial cells, we detected significant downregulation of *p38/Mapk*, MAP kinase-activated protein kinase 2 (*Mk2*), and *Mk3* genes in infected host cells. Suppression of MAPK signaling was associated with an impaired intestinal epithelial defense against *C. parvum* infection. Our data suggest that cryptosporidial infection may suppress intestinal epithelial cell MAPK signaling for evasion of host antimicrobial defense.

Collective ERK/Akt activity waves orchestrate epithelial homeostasis by driving apoptosis-induced survival

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Cell death events continuously challenge the epithelial barrier function, yet are crucial to eliminate old or critically damaged cells. How such apoptotic events are spatio-temporally organized to maintain epithelial homeostasis remains unclear. To study single-cell signaling dynamics we used two fluorescent biosensors for Extracellular Signal-Regulated Kinase (ERK) and AKT serine/threonine kinase (Akt), two important pro-survival signaling nodes. Thanks to our image analysis pipeline we collected the signaling activity status of each cell in the epithelial community. We observed waves of ERK and Akt activity pulses that originate from apoptotic cells and propagate radially to healthy surrounding cells. Such a propagation requires Epidermal Growth Factor Receptor (EGFR) and matrix metalloproteinase (MMP) signaling. At the single-cell level, ERK/Akt waves act as spatial survival signals that locally protect cells in the vicinity of the epithelial injury from apoptosis. By using convolutional neural networks, automatic identification of collective events, fate tracing of neighboring cells and optogenetics we identified a 3-4 hours survival period after each ERK/Akt activity pulse. At the cell population level, the sum of multiple ERK/Akt waves allows the epithelium to maintain homeostasis in response to mild or intense insults, such as the addition of a chemotherapeutic drug. Disruption of this spatial signaling system results in the inability of a model epithelial tissue to guarantee barrier function in response to cellular stress. Our study shows that a simple single-cell ability to communicate and respond to ERK/Akt activity pulses
generates a tissue-level emergent property of coordinating apoptotic events to ensure epithelial homeostasis.

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Signaling diversity enabled by Rap1-regulated plasma membrane ERK with distinct temporal dynamics

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A variety of different signals induce specific responses through a common, extracellular-signal regulated kinase (ERK)-dependent cascade. It has been suggested that signaling specificity can be achieved through precise temporal regulation of ERK activity. Given the wide distribution of ERK substrates across different subcellular compartments, it is important to understand how ERK activity is temporally regulated at specific subcellular locations. To address this question, we have expanded the toolbox of Förster Resonance Energy Transfer (FRET)-based ERK biosensors by creating a series of improved biosensors targeted to various subcellular regions via sequence specific motifs to measure spatiotemporal changes in ERK activity. Using these sensors, we show that EGF induces sustained ERK activity near the plasma membrane in sharp contrast to the transient activity observed in the cytoplasm and nucleus. Furthermore, EGF-induced plasma membrane ERK activity involves Rap1, a noncanonical activator, and controls cell morphology and EGF-induced membrane protrusion dynamics. Our work strongly supports that spatial and temporal regulation of ERK activity is integrated to control signaling specificity from a single extracellular signal to multiple cellular processes.

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Combining optogenetics with network perturbations highlights different regulatory mechanisms of the ERK signaling pathway

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Mitogen-Activated Protein Kinase (MAPK) signaling networks enable cells to convert extra-cellular inputs such as a growth factor binding to receptor tyrosine kinases (RTKs) into specific cellular outputs. This occurs through production of dynamic ERK activity states that are encoded in the network structure. Despite numerous studies, we still miss crucial information about how different molecular components are wired with a specific network topology that can interpret different input strengths and durations to produce different dynamic ERK states. To explore the MAPK signaling network topology in a scalable fashion, we have built synthetic circuits that consist of an optogenetic RTK (optoFGFR1) or GEF (optoSOS) allowing us to activate ERK at different levels in the MAPK cascade with light pulses of different intensities, durations and frequencies. Single-cell ERK dynamics is then recorded using a spectrally compatible biosensor (ERK-KTR). Combined with automated live imaging and image analysis, our system provides the opportunity to investigate ERK dynamics in response to perturbations with an unsurpassed throughput. We use this system to explore how multiple inputs (soluble GFs + light inputs) compete to control ERK dynamics and to perform a siRNA screen targeting 50 components of the MAPK
network. We observe that the latter is extraordinarily resilient against these perturbations. Performing this screen under different stimulation conditions and in response to activation at the level of the receptor (optoFGFR1) or at the level of Ras (optoSOS) suggests sources of robustness that originate from different feedbacks in the cascade. We also identify the adaptor Grb2 as a weak node in the MAPK cascade, of which perturbation can more efficiently shut down ERK activity than other nodes. Due to its scalability, our system allows for comprehensive analysis of the MAPK network.

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A rate threshold mechanism regulates MAPK stress signaling and survival
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All cells employ signal transduction pathways to respond to changes in extracellular stimulus concentration. Physiologically relevant changes in many different stimuli vary as functions of both concentration and rate in healthy and diseased states. Switch-like changes in stimulus concentration show that the strength of signaling and overall cellular response is dependent on stimulus concentration, which in many cases needs to exceed a certain threshold. However, how stimulation rate influences signaling and cell behavior remains poorly understood. Here, we show that budding yeast high osmotic stress survival and mitogen-activated protein kinase (MAPK) signaling depend on a rate threshold. We found that this rate threshold operates to determine the timing of MAPK activation during rate-varying stimulus treatments. We also discovered that the stimulation rate threshold and cell viability is sensitive to changes in the expression levels of the Ptp2 phosphatase, but not of another phosphatase that similarly regulates osmostress signaling during switch-like treatments. Our results demonstrate that the stimulation rate is a regulated determinant of signaling output. We anticipate our approach to provide a blueprint for dissecting primary stimulation rate-dependent regulatory mechanisms in other systems. Such mechanisms may have disease relevance due to the potential alteration of the stimulation rate required for signaling by mutant signaling proteins.

New Techniques in Single Molecule and Super-Resolution Microscopy

P774

Spying on viruses: translation and replication dynamics of single RNA viruses
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RNA viruses are among the most prevalent pathogens and represent a major burden for society. While RNA viruses have been studied extensively, very little is known about the processes that occur during the first several hours of infection due to a lack of sensitive assays. We developed a single-molecule imaging assay, virus imaging (VIRIM), to directly observe and quantify infection, translation and replication of individual RNA viruses in live cells. VIRIM uncovered a striking heterogeneity in replication dynamics between cells, and revealed extensive coordination between translation and replication of
single viral RNAs. Furthermore, using VIRIM we identify the replication step of the incoming viral RNA as a major bottleneck of successful infection, and identify novel host genes that are responsible for inhibition of early viral replication. Single-molecule imaging of virus infection represents a powerful tool to study virus replication and virus-host interactions, and may be broadly applicable to RNA viruses.

P775

**Application of metal-enhanced fluorescence for imaging of biological systems.**

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Fluorescence microscopy is a highly sought-after tool in the field of cell biology for the control and ease it provides in imaging of live and fixed cells, therefore, identification and observation of various novel processes and targets in real-time. The extinction coefficient, quantum yield and fluorescence lifetime of fluorophores all contribute to their utility for fluorescence-based applications. Despite the versatility of fluorophores their brightness can be dampened from factors like photobleaching, instability and phototoxicity. Such limitations can be mitigated by metal-enhanced fluorescence (MEF), yet the application of MEF to fluorescence imaging in cell biology remains limited. MEF occurs when the electron cloud of colloidal metal interacts with fluorophores dramatically changing their spectral properties. Colloidal metal decreases fluorophore lifetime while enhancing fluorophore brightness by increasing the population of excited fluorophores and altering the rate of radioactive decay. Thus, by using MEF the range of fluorophores used in single molecule studies may be expanded, and spatiotemporal resolution of imaging of molecules in signaling pathways, cell membrane traffic and organelle dynamics may also be improved. We aim to develop and optimize the use of silver nanoparticles (AgNP) for fluorescence imaging of cells in culture to improve the detection of fluorophores. To do so, we first performed a series of chemical characterization experiments involving AgNP and fluorophores, both in solution and embedded within a layer of gelatin. We analyzed AgNP of three different shapes and sizes for MEF using BODIPY, and found that the presence of AgNP significantly enhances fluorophore brightness. This was essential for our understanding of how AgNP-BODIPY interaction might alter based on their distribution inside cells with significantly complex spatial organization. We then examined how MEF using AgNP and BODIPY-labelled molecules could enhance fluorescence imaging of single particles at the cell surface and specific organelles in living cells in culture. As an example, we found that AgNP enhanced fluorescence of BODIPY-labelled lysosomal markers in RAW 266.7 macrophages, without appreciably impacting cell morphology and physiology. We expect that these studies will allow the application of MEF in fluorescence imaging as this strategy may improve signal-to-noise for a range of fluorophores.

P776

**Three-dimensional reconstructions of bacterial cells reveal geometric enrichment of cytoskeletal and periskeletal proteins**

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Bacteria cell shape is important for how cells interact with their environment. We use a computational imaging framework to reconstruct the 3D shape of individual cells from fluorescence images and determine where a secondary fluorescent probe is enriched. This computational imaging framework has support for multiple imaging modalities including wide-field fluorescence and SIM imaging with a resulting reconstruction resolution of <50 nm. In addition to characterizing the distribution of individual cell shapes, we have turned our attention to the cell shape associated cytoskeletal proteins. These include the actin-like MreB in straight-rod *Escherichia coli*, the curvature determinants CrvAB in bent-rod *Vibrio cholerae*, and the bactofilin CcmA in helical-rod *Helicobacter pylori*. In *E. coli*, MreB forms elongated structures that are preferentially localized to areas with low Gaussian curvature, avoiding the cell poles. This geometric enrichment, and MreB polymer number, are modulated by the transmembrane protein RodZ. Changes in these parameters are predictive of the resulting changes in cell shape ($r^2=0.93$). Our data suggest RodZ promotes the assembly of geometrically localized MreB polymers that lead to the growth of uniform cylinders. Shapes that are more complex than a straight rod have an added twist to their story and require additional protein determinants. We further study localization of the shape determining proteins in the helical-rod shaped human pathogen *Helicobacter pylori*. *H. pylori* localizes the bactofilin CcmA along the outer curve. Strains expressing CcmA variants with altered polymerization properties lose helical shape. We thus propose a model where CcmA and MreB promote cell wall synthesis at positive and negative Gaussian curvatures, respectively, and that this patterning is one mechanism necessary for maintaining helical shape. These biological discoveries have been enabled by our development of quantitative, precision microscopy technologies that enable us to resolve the sub-micron, three-dimensional shape of bacterial cells.

**Matrix - an array-based detector for background reduction with Sted microscopy**

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STED microscopy is a well-established method to improve the spatial resolution of scanning fluorescence microscopes. Nevertheless, improving spatial resolution results in a reduction of the volume of the effective point-spread-function and thus images that appear dimmer. Since STED mostly has an effect in or close to the focal plane, this means that signal seemingly decreases there, while background signal from all throughout the sample still has the same strength as in conventional confocal microscopy. In other words, the signal-to-background ratio often decreases when doing STED on samples that are thicker than a few microns. Here, we present a method to sense and subtract background for each individual image pixel using an array-based detector. This type of detector has been used together with methods such as “pixel reassignment” or the “Sheppard sum” for increasing the resolution to that of a small pinhole, while collecting photons over an area corresponding to an open pinhole. We show that additionally, an array-based detector can be harnessed to discriminate in-focus signal from out-of-focus background using only a single 2D (xy) image from the sample. This significantly increases optical sectioning and signal-to-background ratio, and enables high-resolution STED microscopy on thick structures. To demonstrate the advantages offered by MATRIX detection, we
present super-resolution STED images of several biologically relevant samples which showcase superior signal-to-noise over conventional STED microscopy.

P778

**Cryogenic correlative single-molecule fluorescence localizations and electron tomography reveals bacterial subcellular organization**


Organization of biomolecules in bacteria is critical to their survival and adaptation. However, the nanoscale observation of this organization is challenging. Super-resolution (SR) fluorescence microscopy and cryogenic electron tomography (CET) are two powerful microscopy methods for observing the subcellular organization of biomolecules, but each approach has limitations. SR fluorescence microscopy utilizes attachment of specific proteins to fluorescent labels allowing for a SR image of a specific protein *in situ*, but the SR reconstructions lack cellular context beyond the proteins that have been labelled. CET on the other hand, provides molecular scale resolution, but the vast majority of proteins are not discernable in the reconstructions and the method lacks specific and non-perturbative labelling methods. Here I will describe a combination of cryogenic photoactivated localization microscopy (PALM) based SR and CET that provides precise and accurate localizations from fluorescence to annotate indiscernible proteins in high-resolution CET reconstructions. We call the approach Correlative Imaging by Annotation with Single Molecules or CIASM. Specifically, CIASM achieves single-molecule localizations with an average lateral precision of 9 nm, and the SR and CET datasets can be aligned with a relative error of ~30 nm. I will demonstrate our method in the model organism *Caulobacter crescentus* by annotating the positions of three proteins: McpA, PopZ, and SpmX. McpA, which forms a part of the chemoreceptor array, acts as a validation structure by being visible under both imaging modalities. In contrast, PopZ and SpmX, are a part of a polar membraneless organelle and cannot be directly identified in CET. While not directly discernable, PopZ fills a region at the cell poles that is devoid of electron-dense ribosomes. We annotate the position of PopZ with single-molecule localizations and confirm its position within the ribosome excluded region. We further use the locations of PopZ to provide context for localizations of SpmX, a low-copy integral membrane protein sequestered by PopZ as part of a signaling pathway that leads to an asymmetric cell division. Our correlative approach reveals that SpmX localizes along one side of the cell pole and its extent closely matches that of the PopZ region.

**Nuclear Envelope Rupture and Sealing**

P779

**Investigating post-mitotic nuclear envelope sealing mechanisms in fission yeast**

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The nuclear envelope maintains compartmentalization between the nucleoplasm and the cytoplasm. In an “open mitosis”, the nuclear envelope breaks down to allow the spindle access to chromosomes and ensure their segregation to daughter cells. Nuclear compartmentalization is re-established at mitotic exit through an extremely complex choreography of molecular events that remain to be fully defined.
but ultimately require the sealing of nuclear envelope holes. In an effort to home in on the molecular players and mechanisms that seal the nuclear envelope in a more simplified system, we are investigating the closure of a single nuclear envelope hole formed after the extrusion of the spindle pole body (SPB) from the nuclear envelope of the fission yeast, *Schizosaccharomyces pombe*. This event occurs at the end of every mitosis and leaves a ~200 nm hole that must be closed in order to maintain nuclear compartmentalization. Our data support a model in which the endosomal sorting complexes required for transport (ESCRT) machinery are recruited to sites of SPB extrusion in a sequential order beginning with Cmp7, the orthologue of CHMP7. We will present data that leverages live-cell imaging of fluorescently-tagged sealing factors, correlative light and electron tomography, and conditional degradation approaches to illuminate the function of putative sealing factors. As ESCRTs seal the nuclear envelope during post-mitotic nuclear envelope reassembly in mammalian cells, our data will directly inform the mammalian system and provide needed molecular insight into a critical, but ill-defined, cellular process.

P780

**Direct PA-binding by Chm7 is required for nuclear envelope surveillance at herniations**

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There is considerable evidence to support that the integrity of the nuclear envelope membranes and the function of nuclear pore complexes (NPCs) are surveilled by components of the endosomal sorting complexes required for transport (ESCRT). The precise mechanisms that contribute to ESCRT recruitment, activation and membrane remodeling at the nuclear envelope remain to be fully defined. We will present data that supports a key role for the local accumulation of phosphatidic acid (PA) in contributing to nuclear envelope recruitment and function of the budding yeast ESCRT, Chm7 (orthologue of CHMP7). Using direct in vitro binding with recombinant Chm7 and liposomes of defined lipid compositions, we will show a direct and specific PA-binding activity of this ESCRT. This PA-binding is mediated through a conserved hydrophobic stretch of amino acids, which confers specific binding to the nuclear envelope in genetic backgrounds where PA levels are elevated. Further, these PA-specific nuclear envelope interactions are independent but are nonetheless required for interaction with the LAP2-emerin-MAN1 (LEM) domain protein, Heh1 (LEM2). Consistent with the functional importance of PA-binding for nuclear envelope surveillance, mutation of the PA-binding motif inhibits recruitment of Chm7 to the nuclear envelope and abolishes Chm7 function in genetic backgrounds where NPC-assembly-associated nuclear envelope herniations or “blebs” occur. In fact, we show using correlative light and electron tomography that a fluorescent PA sensor specifically accumulates at sites of these nuclear envelope herniations. Thus, we suggest that local control of PA metabolism is important for ensuring productive nuclear envelope remodeling by ESCRTs and that its dysregulation may contribute to pathologies associated with defective NPC assembly.
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Barrier-to-autointegration factor controls dynamics of nuclear leakage and repair following nuclear envelope rupture

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The nuclear envelope (NE) provides a critical barrier between the cytosol and nucleus that is key for cellular compartmentalization and protecting genomic DNA. NE rupturing during interphase been implicated in several NE-associated diseases and cancers. However, NE ruptures are repairable, and recent studies have revealed the highly mobile DNA-binding protein barrier-to-autointegration factor (BAF) as a major participant in the NE repair process. Upon NE rupture, cytosolic BAF rapidly binds to nuclear DNA exposed to the cytosol and subsequently recruits components to repair the NE, including LEM-domain proteins, lamins, membranes, and ESCRT-III repair machinery. To further examine the mechanisms by which BAF aids in repairing NE ruptures, we expressed BAF variants with localization motifs or amino acid substitutions that modify its functions in human fibroblasts depleted of endogenous BAF and analyzed the dynamics of NE rupture repair. We found that restricting BAF to the nucleus exacerbated nuclear leakage and prevented rupture repair, while restricting BAF to the cytoplasmic compartment enabled efficient rupture repair, supportive of an essential role for cytoplasmic BAF in repairing NE ruptures. Expression of BAF variants with a reduced affinity for DNA or LEM-domain binding also inhibited NE rupture repair, demonstrating the requirement of these interactions in the NE repair process. Unexpectedly, we observed that loss of lamins enables the repair of NE ruptures in BAF-depleted cells. Our ongoing studies seek to further understand the mechanisms by which BAF regulates rupture-induced nucleocytoplasmic exchange and the repair of NE ruptures, and mechanisms by which the nuclear lamina prevents rupture repair in BAF-depleted cells.

P782

LMNA-Mutant Stem Cell-Derived Cardiomyocytes Exhibit Nuclear Defects, Lamin Mislocalization, and Altered DNA Damage Response

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The nuclear lamina is a meshwork of lamin proteins inside of the inner nuclear membrane that gives the nucleus structural support, organizes peripheral chromatin, and plays a role in diverse intracellular signaling. Lamins A and C are encoded by the \textit{LMNA} gene, and mutations in this gene cause a collection of diseases termed laminopathies that include dilated cardiomyopathy (\textit{LMNA}-DCM) and skeletal muscular dystrophies. On the cellular level, skeletal muscle laminopathies are characterized by structural defects resulting from mechanically weakened nuclei and disruption of signaling pathways. It remains unclear how nuclear mechanical damage plays a role in \textit{LMNA}-DCM. Herein, we use induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) from healthy individuals and two \textit{LMNA}-DCM patients (R249Q, G449V) to examine nuclear damage, and its causes and consequences to cellular
function. Compared with control iPSC-CMS, R249Q-iPSC-CMs had abnormal nuclear shape, increased nuclear area, and increased nuclear volume, while G449V-iPSC-CMs had only increased nuclear area, consistent with nuclear flattening. Nuclear deformities suggest altered nuclear mechanics, which can render the nucleus prone to nuclear envelope (NE) rupture. Using a NE rupture reporter system, we observed increased NE rupture in R249Q-iPSC-CMs compared to controls, consistent with the more severe defects in nuclear mechanics. G449V-iPSC-CMs showed only a slight, non-significant increase over controls. LMNA mutations could alter Lamin A/C self-assembly, phosphorylation or stability, resulting in improper lamin assembly and organization, and altered nuclear mechanics. R249Q-iPSC-CMs, and to a lesser extent G449V-iPSC-CMs, had decreased peripheral localization and increased nucleoplasmic Lamin A/C staining, indicating disturbed Lamin A/C assembly. As DNA damage and defective DNA damage response (DDR) have been described in other LMNA mutations, potentially as a result of NE rupture, we quantified γH2AX and recruitment of 53BP1, a protein critical for DNA repair that interacts with Lamin A/C. While basal levels of DNA damage in the LMNA mutant iPSC-CMs were similar to controls, 53BP1 recruitment in response to Phleomycin-induced DNA damage was severely impaired in both R249Q and G449V-iPSC-CMs. Together, these results indicate altered nuclear mechanics in LMNA-DCM, likely due to altered Lamin A/C assembly, that impacts nuclear structural integrity and potentially downstream signaling, including DDR activation. Future work will be directed at understanding the mechanisms of Lamin A/C mislocalization, altered NE mechanics, impaired DDR, and how these defective processes contribute to cardiac muscle defects observed in laminopathies, which will guide development of therapies.

P783

Evaluating the contribution of nuclear lamins and the LINC complex to nuclear ruptures

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The most common tool to study nuclear rupture and repair is a fluorescent protein fused to nuclear or cytoplasmic targeting sequence. Our studies with GFP-3NLS revealed an inherent limitation of transport-dependent rupture reporters when experimental conditions significantly alter active transport. Therefore, we developed a transport independent Hsp90-GFP rupture reporter. We also have evidence that focal laser exposure at the nuclear envelope does not immediately generate nuclear ruptures, but instead weakens the nuclear envelope and its associated structures that eventually lead to a nuclear rupture of variable size. We hypothesize that this variation in rupture size in part reflects the balance of forces acting on the nuclear envelope. Here, we used Hsp90-GFP in combination with laser-induced nuclear rupture to study the steady-state force balance between the nucleus and the cytoskeleton mediated by the nuclear lamina and the LINC complex. We observed, as reported previously, that loss of A-type lamins leads to larger ruptures, presumably by weakening the nuclear lamina and increasing susceptibility to cytoskeletal forces. Complete disruption of the LINC complex by co-depletion of Sun1 and Sun2 does not appreciably increase the extent of leakage, but does delay repair. Co-depletion of the LINC complex and A-type lamins leads to nuclear leakage far more extensive than observed by loss of A-type lamins-alone. However, co-depletion of individual LINC complex proteins Sun1 or Sun2 corrects the enhanced rupture caused by loss of A-type lamins. Together, these data suggest that laser-induced nuclear rupture may enable study of how the LINC complex and lamina synergistically function to regulate the force balance between the nucleus and cytoskeleton during interphase. Ongoing studies
are evaluating how individual nesprins, cytoskeletal forces and chromatin regulation might be contributing to these ruptures.

P784

**Nuclear envelope-associated progeria perturbs targeting of A-type lamins to nuclear ruptures**

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Mutations in three genes encoding nuclear envelope (NE) proteins, *LMNA, BANF1*, and *LEMD2*, result in accelerated aging syndromes known as progerias. These proteins interact with each other to accomplish various cellular processes. One of these functions is the repair of interphase nuclear ruptures to ensure the efficient reconstitution of the nuclear-cytoplasmic barrier. Previously, we have shown that barrier-to-autointegration factor (BAF) localizes to sites of nuclear rupture and is required for recruiting NE-repair machinery, including the LEM-domain proteins, ESCRT-III complex, and membranes. Here, we show that a mobile, nucleoplasmic population of A-type lamins is recruited to ruptures in a BAF-dependent manner via BAF's association with the Ig-like domain of A-type lamins. A progeria-associated BAF mutant targets to nuclear ruptures; however, it is unable to recruit A-type lamins. Multiple progeria-associated lamin A (LaA) mutations inhibit its recruitment to nuclear ruptures. The permanently farnesylated progeric LaA-Δ50 does not accumulate at ruptures, perhaps due to decreased nucleoplasmic localization. The progeric LaA-K542N mutation, structurally predicted to disrupt its association with BAF, also did not localize to nuclear ruptures. Together, these data support a model where defective localization of A-type lamins at nuclear ruptures could be a shared mechanism of NE-associated progeria, perhaps by enhancing the propensity for nuclear rupture and/or by compromising nuclear rupture repair which contribute to progeria phenotypes, including increased DNA damage and cellular senescence. Ongoing studies are assessing the functional consequences of progeria mutations in BAF and A-type lamins during the nuclear rupture and repair process.

P785

**Chromatin composition determines micronuclei membrane integrity**

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Chromosome(s) that lag due to mitotic errors recruit nuclear membrane to form micronuclei (MN), distinct cellular compartments from the primary nucleus (PN). Compared to PN in the same cell, MN membranes are highly unstable and prone to irreversible rupture. MN rupture can lead to aneuploidy, genome instability, and trigger innate immune and tumorigenic signaling. Therefore, identifying the factors that contribute to MN membrane stability is essential to preventing rupture events and cancer evolution. The integrity of the nuclear lamina, a major source of nuclei structural support, has been shown to contribute to MN stability. MN have reduced Lamin B1 levels compared to the PN in the same cells and rupture occurs at gaps in the lamina, however the origin of these lamina defects remains unclear. Chromatin compaction state is another critical nuclear membrane/lamina recruitment factor and contributes to PN structure. Yet, the contribution of chromatin content and compaction to MN lamina formation and membrane integrity has yet to be identified. To understand the contribution of chromatin, we assessed micronucleus stability for 10 chromosomes with a spectrum of intrinsic features including length, centromere size, and gene density in human cells by IF-FISH. We determined that MN
rupture frequency varied widely between individual chromosomes, indicating that chromosome identity influences stability. We identified a positive correlation between membrane stability and both chromosome length and gene density. We next assessed lamin protein recruitment and found that Lamin B1 levels are determined by MN size however, gene density does not correlate with Lamin B1 recruitment. This suggests an alternative mechanism for establishing membrane stability for small gene dense chromosomes. MN lamina may not fully explain membrane integrity and the chromatin itself may be playing a role in membrane stability. We are currently working to test the hypothesis that differences in replication timing, transcription levels, DNA compaction, and/or chromatin tethering to the nuclear lamina account for the increased membrane stability associated with euchromatic micronuclei.

P786

Tracking micronucleated chromatin

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Following mitotic errors, one or more chromosomes or chromatin fragments may form separate nuclear compartments from the primary nucleus, called micronuclei. Though micronuclei are grossly similar in structure to primary nuclei, they are prone to irreversible membrane rupture. This exposes the chromatin trapped within to the cytosol for the rest of interphase. Micronuclei have long been recognized as indicators of chromosome instability and DNA damage, but in recent years have also been found to drive genome instability—specifically chromothripsis, innate immune signaling, and metastasis. However, it is likely that many of the consequences of prolonged cytosolic exposure of chromatin are still unknown. To investigate the possible consequences of ruptured micronuclei, I am developing a tool based on the bacterial enzyme DNA adenosine methyltransferase (Dam) to selectively label chromatin exposed by micronucleus rupture. Dam methylates adenosine into N-6-methyladenosine (m6A), a unique long-lived covalent mark in mammals that, when expressed at low levels, does not interfere with normal cellular processes. Tracking m6A-labelled chromatin would allow me to follow chromatin from ruptured micronuclei over multiple cell cycles. To selectively label chromatin in ruptured micronuclei, Dam activity is restricted to interphase cytosol by tagging it with a nuclear exclusion signal and increasing its size to exclude it from nuclear compartments, and regulated by a TetOn inducible promoter and conditional degron domain to give tight control over when Dam is active. Analyses of Dam activity by PCR and immunofluorescence of m6A DNA demonstrate that this system produces sufficient Dam expression to label ruptured micronuclei while keeping activity low enough to minimize background genomic methylation. I am continuing to optimize and validate this system, with the goal of being able to selectively label micronuclei that have ruptured in different phases of the cell cycle. In addition, I am optimizing the expression of a dead DpnI enzyme, which selectively binds m6A, to facilitate visualizing micronuclear rupture in living cells. Together, these tools will allow me to interrogate the consequences of micronucleus rupture, including long-term functional changes to affected chromatin, the kinetics of downstream inflammatory responses, and whether and how micronucleus rupture triggers cell cycle arrest. In addition, this tool would aid in understanding other processes that generate cytosolic DNA, from chromatin bridge resolution to DNA virus infection and immunity.
Physical Approaches to Cell Biology 3

P787

Mechanical constraints play a critical role in protein segregation and morphogenesis in the early C. elegans embryo

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During development, cells take on specific fates to properly build tissues and organs. These cell fates are regulated by a number of factors, including biochemical signaling, mechanical constraints, and cell geometry. However, it is not yet understood how these factors work together to regulate patterning during development, and how the same interactions can lead to different responses to perturbations. Exploiting the high conservation of developmental pathways and processes, we theoretically and experimentally explore mechanisms of protein patterning and cell fate specification during development of the nematode worm C. elegans. Biologically based mathematical models identify key components and interactions required for wild type patterning, which can differ when the model is subject to mechanical perturbations. Testable hypotheses based on model results are used to investigate protein patterning and cell fate specification in both wild type and perturbed conditions, revealing how the same underlying interaction network can give rise to different behaviors under certain conditions. The rich data sets produced by these models form the basis for further analysis and increase our understanding of cell fate regulation in development.

P788

Influence of Force on the E. coli Adhesin, FimH

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Mechanical forces influence functions occurring inside and around cells. Forces have influenced some proteins to develop catch bond properties, in which the lifetime between a biomolecule and ligand increases with applied force. Catch bonds have been observed in various proteins such as cell and bacterial adhesive proteins. One such bacterial adhesive, FimH, allows E. coli to resist strong shear forces found in high flow environments of the intestinal or urinary tract to facilitate infection into host cells. The published lifetimes under force of most catch bonds are typically on the order of seconds. While the full lifetime of FimH under force has never been fully measured, preliminary measurements indicate that the lifetime of the FimH catch bond far exceeds that of other catch bonds. Therefore, it is suspected that FimH possesses an unusually strong catch bond property. The goal of this study is to measure the lifetime of FimH under force to compare the strength of this catch bond to other catch bonds and eventually understand the mechanism that gives FimH its unusual strength under force. To facilitate the measuring of long interactions under force, we utilized a novel magnetic tweezer apparatus that can apply constant stable forces to multiple magnetic beads simultaneously. Magnetic beads coated in mannose ligand were manipulated via electromagnets to form bonds with immobilized FimH. After the catch bond was activated by a preload force, the tension on the bond was held constant until the bead was observed to detach from the surface. At forces up to 70 pN, many FimH bonds outlasted the 15-minute collection period. In fact, we estimated lifetime of the bond under 20-30 pN of
force to be about 17 minutes. In contrast, other catch bond forming proteins such as, integrins, cadherins and P-selectins, last less than 10 seconds and the high affinity bond between biotin and streptavidin unbinds within a second within the same force range. Like other catch bonds, once the FimH catch bond is activated, increasing force negatively affects the bond lifetime. However, when the change in unbinding rate due to force was estimated, we found that FimH exhibited a relatively force insensitive behavior. We believe this relative force insensitivity contributes to FimH’s remarkably long lifetime under force.

P789

**Three-dimensional niche stiffness synergizes with WNT7a to modulate the extent of satellite cell symmetric self-renewal divisions**

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Characterising the mechanisms of endogenous muscle regeneration and recapitulating features in an *in vitro* setting are critical for developing effective regenerative therapies for muscle wasting conditions. While many signalling pathways are known to control muscle stem cell (satellite cell/SC) self-renewal, less is known about the mechanisms underlying their spatiotemporal control. Here, we measured biomechanical changes that accompany skeletal muscle regeneration and determined the implications on SC fate. Using atomic force microscopy, we quantified a 2.9-fold stiffening of the SC niche at time-points associated with planar-oriented symmetric self-renewal divisions. Immunohistochemical analysis confirms increased extracellular matrix deposition within the basal lamina. To test whether three-dimensional (3D) niche stiffness can alter SC behaviour or fate, we embedded isolated SC-associated muscle fibres within biochemically inert agarose gels tuned to mimic native tissue stiffness. Time-lapse microscopy revealed that a stiff 3D niche significantly increased the proportion of planar-oriented divisions, without effecting SC viability, fibronectin deposition, or expression of the cell fate determining proteins PAX7 and MYOD. We then found that 3D niche stiffness synergizes with WNT7a, a biomolecule shown to control SC symmetric self-renewal divisions via the noncanonical WNT/planar cell polarity pathway, to modify stem cell pool expansion. Here, we present a novel model to modulate niche stiffness without altering biochemical cues using isolated myofibre cultures, and provide new insights into the role of 3D niche biomechanics in regulating SC fate choice.

P790

**Understanding stochastic breakdown in tissue structure during breast cancer progression through the lens of statistical mechanics**

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Structural breakdown of the mammary epithelium, a normally bilayered tissue comprising an inner luminal (LEP) and outer myoepithelial (MEP) layer, is common to all breast cancers subtypes. Progression to invasive disease occurs when transformed LEP translocate past MEP, and represents a major inflection point in risk. LEP are normally prevented from translocating by active self-organization that maintains MEP at the outer edge of the tissue. How this process breaks down to permit invasion is not understood. We present a statistical mechanical framework to characterize how interfacial energies, positional entropy, and tissue activity (analogous to temperature) collectively determine the probability distribution of tissue structure in healthy and diseased mammary organoids. Organotypic cultures of patient-derived human mammary epithelial cells self-organize in vitro, largely driven by differences in favorability of LEP and MEP interface with the extracellular matrix. The observed distribution of tissue structures closely aligns with Boltzmann statistics - a function of the underlying interfacial energies (enthalpy), geometric constraints (entropy), and mechanical fluctuations (activity) of the tissue. We predict that transformations which increase the probability of LEP occupancy in the basal compartment can destabilize tissue structure and promote invasion, consistent with observations in murine organoid models. To test these predictions experimentally, we examined the ability of 15 cancer-associated genetic changes to alter interfacial tensions of LEP and disrupt self-organization in reconstituted human mammary organoids. Most perturbations had only minimal impact on self-organization, including knock-down of E-cadherin. In contrast, PIK3CA activation in LEP uniquely reduced their ECM interfacial energy and disrupted tissue structure. Modeling predicts that normalization of PIK3CA-LEP interfacial energy or decreasing overall tissue activity correct tissue structure, which we confirm experimentally. RNA-seq analysis revealed that PIK3CA-LEP upregulate a basal adhesion program, a signature also observed in mouse models of PIK3CA-driven breast cancer and during the progression of in situ to invasive lesions in humans. These studies suggest new molecular markers for predicting disease progression, new strategies for preventing structural loss during cancer progression, and provide a new framework for understanding the heterogeneity in tissue structures observed during development, regeneration, and disease.

P791

Physical Basis for Epithelial Cell Reintegration

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Proliferative epithelia face a challenge: tissue integrity must be maintained despite the potentially disruptive process of cell division. One way for tissues to address this challenge is by allowing dividing nuclei to move apically, freeing them from physical constraints (crowding) imposed by their neighbors. Our work addresses epithelial cell reintegration, the mechanism by which newly-born, apically-positioned daughter cells reincorporate basally. Reintegration depends on both lateral adhesion between cells as well as the ability to transmit force through adhesion to the cytoskeleton. In Drosophila, the lateral adhesion protein Neuroglian works through the molecular clutch Ankyrin to connect to the cytoskeleton. The process of axon growth and pathfinding works through the same physical mechanism, suggesting a functionally conserved assembly. We hypothesize that reintegration requires the adhesive force that draws the cell basally to overcome a resistive force from the underlying epithelium. To test this possibility, I am investigating the role of layer stiffness and cell contractility in reintegration by coupling vertex based and rheological computational modelling with light microscopy. I
have created a 2D model of an epithelium in the XZ plane. Cells in this epithelium have multiple lateral adhesion binding sites, which when close enough to a neighboring cell, form lateral adhesions and allow for complex lateral shape change. Confocal microscopy is being paired with a custom-built cell stretching device to mechanically alter cell properties. Together, these approaches help to determine how basic physical attributes of a proliferative epithelium affect maintenance and development.

P792

Towards unraveling the role of mechanical cues in B cell activation

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Generation of antigen-specific antibodies by B cells is crucial for protection against diseases and provides the basis for successful vaccination. Rational design of new vaccines requires in-depth mechanistic understanding of how B cells recognize and discriminate antigens to trigger antigen-specific antibody responses. Mechanotransduction has been recently demonstrated to play a critical role in B cell antigen recognition and activation via the B cell receptor (BCR). However, there is a critical gap in our understanding of the events leading from the initial BCR-antigen binding event to the initiation of intracellular signalling. A challenging aspect of this problem is that B cells integrate a number of chemical, mechanical, and spatial cues at the molecular level when deciding whether or not to respond to a particular antigen. Therefore, biophysical approaches capable of quantitative and reproducible control of B cell activation will be key in validating the different mechanisms that have been proposed so far. Our approach combines single-molecule fluorescence microscopy, Ca²⁺ imaging, and DNA-based molecular force sensors to determine how BCR binding to membrane-tethered antigen initiates intracellular signalling, and how chemical and physical cues such as antigen affinity and mechanical tension regulate this process. We are resolving and tracking the formation of individual BCR-antigen bonds, while simultaneously quantifying intracellular signalling and controlling the mechanical signals transmitted to the BCR. This enables us to capture the transition from the B cell resting state to an activated state, providing new insight into the mechanisms of antigen-triggered B cell activation. Our ultimate goal is to develop mathematical models to predict how B cells will respond to an antigen stimulus received in a particular mechanical environment, which may catalyze new approaches in vaccine development.

Protein Dynamics in Neurons

P793

Neuronal polarity requires dendritic protein endocytosis in the axon initial segment

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Neurons are highly polarized cells that must compartmentalize a vast repertoire of proteins to either the axon or dendrites, a neuron’s morphologically, biochemically, and functionally distinct domains. The axon initial segment (AIS) is a critical region between the axon and dendrites that acts as a selective filter for intracellular vesicles and a diffusion barrier to establish and maintain neuronal polarity. How
the AIS functions to establish and maintain strict compartmentalization of proteins remains unresolved. We uncovered a novel, conserved mechanism of neuronal polarity in the AIS in which dendritic transmembrane proteins are prevented from entering the axon through their endocytosis and degradation in the AIS. We identified this mechanism by studying the dendritic morphology guidance receptor, DMA-1, which is required for the *C. elegans* PVD neuron’s elaborate dendritic branching. Endogenously labeled DMA-1 is highly polarized to the dendrite and excluded from the axon. Using an *in vivo* endocytosis reporter, we unexpectedly find that DMA-1 is highly endocytosed in the AIS, a region previously thought to lack endocytosis due to its dense cytoskeletal network. However, we find that endogenously labeled endocytic proteins, clathrin light chain and AP-2, localize to puncta in the AIS and display characteristic features of endocytic structures. Endocytic inhibition through manipulating AP-2 or dynamin causes mislocalization of DMA-1 to the axon within hours and aberrant axonal branching due to DMA-1’s function in neurite branching, thus demonstrating a functional role for AIS endocytosis in neuronal polarity. We identify two DMA-1 endocytic interaction mechanisms that together select and ensure efficient DMA-1 endocytosis in the AIS: a cytoplasmic interaction with AP-2 and an extracellular domain interaction with two LRP (low density lipoprotein receptor-related) proteins. After its endocytosis, DMA-1 localizes to late endosomes in the AIS, suggesting that DMA-1 is endocytosed and degraded to prevent its further entry into the axon. Therefore, endocytosis is an active mechanism to remove dendritic proteins from the AIS and maintain neuronal polarity. We further demonstrate that this mechanism is broadly used by diverse dendritic proteins in additional *C. elegans* neurons and is evolutionarily conserved. Using induced human neurons, we find that the dendritically polarized transferrin receptor is endocytosed in the AIS and endocytosis is essential for its polarity. These results define a framework for understanding AIS endocytosis and reveal a novel mechanism of endocytosis in the AIS that is critical for neuronal polarity.

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**Kinetic Monitoring of ATF4 Stress Response in Primary Mouse Neurons**

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The activating transcription factor-4 (ATF4), a downstream product of PERK/elf2α signaling, is recognized as a molecular switch that controls cellular fate decisions between adaptive or apoptotic states as part of the integrated stress response. The relative timescale of the ATF4 translational response remains unexplored in neurobiological relevant contexts. This work presents the development and validation of a molecular tool that allows the kinetic monitoring of ATF4 stress responses in neurons. To address this need, we designed a viral-based ATF4 reporter comprising 384 bp of the initial coding region, including the 5’UTR of human ATF4 as a translational control operator fused to enhanced green fluorescent protein (EGFP). To allow its application in primary neurons and hard-to-transfect cells, we manufactured adeno-associated viral vectors (AAV) of the AAV/DJ serotype as gene delivery vehicles. We found that our ATF4-based reporter based on the translational activity of ATF4 exhibits a 20% basal expression in human cell lines and primary mouse neurons compared to a constitutively expressed reporter protein. Furthermore, by conducting automated time-lapse fluorescent microscopy over >36 h, we were able to discern the distinct temporal profiles of the ATF4 translational stress response in neurons (n > 20,000 cells) subjected to calcium depletion (ATF4max = 48 h) or proteasome inhibition (ATF4max = 36 h) perturbations. We are extending the utility of this AAV-based ATF4 reporter to
understand the temporal dynamics of ATF4 in cellular models of protein misfolding and other neurobiological processes. These results will reinforce the understanding of neuronal stress responses involving increased ATF4 translation and enable a more robust assessment of potential therapeutic strategies targeting the integrated stress response in neurological diseases.

P795

Dysregulated nuclear LMNB1 and impaired nucleocytoplasmic transport in Dystonia patient-derived neurons
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Dystonia is the third most common movement disorder characterized by sustained or intermittent muscle contractions. There is no specific treatment to cure this disease due to its unclear pathological mechanisms. The childhood onset DYT1 dystonia represents the most frequent and severe form of hereditary dystonia, providing an excellent example to understand this disease. DYT1 dystonia is caused by a heterozygous mutation in torsin A (TOR1A), a membrane-embedded ATPase. While animal models provide insights into disease mechanisms, significant species-dependent differences exist since mice with the identical heterozygous mutation fail to show pathology. Here, we model DYT1 by using human patient-derived motor neurons. These neurons with the heterozygous TOR1A mutation show markedly thickened nuclear lamina, disrupted nuclear morphology, and impaired nucleocytoplasmic transport, whereas they lack the perinuclear “blebs” that are often observed in animal models. Importantly, we further uncover that the nuclear lamina protein LMNB1 is specifically dysregulated in expression and subcellular localization. LMNB1 downregulation can largely ameliorate all the cellular defects in DYT1 motor neurons. These results reveal the value of disease modeling with human neurons and provide novel molecular mechanisms underlying DYT1 dystonia and potentially other neurological diseases with impaired nucleocytoplasmic transport. This work was supported by NIH grants (NS092616, NS099073, and NS088095 to C.L.Z.; R21NS112910 to B.D.), and Friends of the ADC and NIH ADC grant (NIH/NIA P30-12300-21 to B.D.) and DoD PRMRP grant (W81XWH2010186 to B.D.).

P796

Preservation of polarity pattern in axonal microtubules - a simulation study
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In developing neurons, molecular motors organize microtubule (MT) filaments into a parallel array that functions as a “railway” for intracellular vesicle transport. An essential feature of the axonal MT array is a nearly uniform polarity pattern in which MTs are oriented with their rapidly growing plus ends away from the cell body. Corruption of this polarity pattern in response to disease or injury is associated with disordered vesicle transport, loss of axon/dendrite differentiation, and neurodegeneration. We previously proposed a polarity sorting mechanism in which cytoplasmic dynein slides MTs with their plus ends leading, resulting in regions of uniform polarity. A combination of computational modeling and live cell imaging supported a primarily dynein-based sorting mechanism, while also implicating several other
molecular players, including competing motors such as kinesin-1 and non-motile microtubule cross-linking proteins. In particular, we demonstrated that microtubule cross-linking proteins such as TRIM46 and PRC1 prevent aberrant sliding of axonal MTs, and that inhibition of these proteins leads to polarity flaws. Here, we present a computational simulation study that extends our previous model in order to investigate hypothetical sources of polarity pattern corruption, including nucleation of short MTs with random orientation, and severing of long MTs into short MTs that are more likely to undergo rotational diffusion into a minus-end-out orientation. We demonstrate that small initial numbers of mal-oriented microtubules can lead to progressive accumulation of MT polarity flaws. We posit that microtubule cross-linking proteins have a role in “error prevention” by reducing the likelihood of “sever and flip” events, and we make several testable predictions of the polarity pattern along the axon that results from inhibition of motor proteins and non-motile cross-linking proteins.

P797

**Tau-mediated regulation of multi-motor cargo transport**

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To understand how Tau regulates axonal transport and how defects lead to neurodegenerative diseases, we performed *in vitro* motility assays on early and late phagosomes. Motor proteins are regulated by cargo-specific adaptor proteins and microtubule associated proteins (MAPs) that together direct cargo transport along microtubules. Tau is a neuronal MAP that is implicated in neurodegenerative diseases collectively known as Tauopathies, including Alzheimer’s disease. In its native form, Tau stabilizes, assembles, and bundles microtubules. Pathogenic forms of Tau perturb the organization of the cytoskeleton and have been linked to defective axonal transport. To understand Tau’s native function in regulating intracellular transport, we will map out the ensemble of motors and adaptors that drive the transport of different cargoes and determine how Tau influences their motility. We hypothesize that the specific set of motors and adaptors on a cargo determine its sensitivity to regulation by Tau. Neuronal cytoskeletons are highly complex and observations in cells are often difficult to interpret. Therefore, we use *in vitro* reconstitution and single molecule studies as a simplified approach to understand axonal transport in neurons. Previously, we revealed that late phagosomes move bidirectionally along microtubules and that Tau increases the fraction of minus-end directed motility. Tau biases the directionality of these cargoes by differentially tuning the processivity of kinesin-1, 2 and dynein, suggesting that plus-end motors on late phagosomes are more sensitive to Tau, which enhance dynein’s processivity. We quantified the number of kinesin-1, 2, 3, and dynein present on early phagosomes using stepwise photobleaching. Preliminary findings show that there are more kinesin-1 compared to kinesin-2 on early phagosomes than late phagosomes. Early phagosomes exhibit diffusive and processive periods of motility with a higher fraction of minus-end directed processive runs. The mean length of plus-end runs is 590 nm with an average velocity of 1.3 um/s. Whereas, minus-end runs have a mean length of 670 nm and an average velocity of 1.2 um/s. In the presence of Tau, the average velocity of plus-end runs decreased to 1.0 um/s while the mean length increased to 790 nm and the average velocity of minus-end runs increased to 1.3 um/s while the mean length was unchanged. These results suggest that Tau influences the set of kinesins uniquely on early phagosomes compared to its regulation of kinesins on late phagosomes. In parallel, we are developing a proteomics approach to identify the motors and adaptors on sorting, early, and late phagosomes. These results will clarify the underlying mechanisms that regulate cargo transport and shed light on Tau’s role in regulating axonal transport.
Tumor Microenvironment: Metabolism and Signaling

P798

**Metabolic Effects of Nitric Oxide Vary Along Hypoxic Gradients in an in Vitro Tumor Microenvironment**

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The solid tumor microenvironment (TME) is heterogeneous. Oxygen and nutrient gradients form within the TME as they diffuse from capillaries and are consumed by cells. The spatial distribution of cellular phenotypes within the TME is poorly understood and difficult to study in standard 2D cell culture, spheroids, and tissue. We therefore use restricted exchange environment chambers (REECs) to impose cell-generated hypoxic gradients on breast cancer cells in 2D monoculture. The hypoxic gradients experienced by cells in REECs are an in vitro model of the hypoxic gradients experienced in vivo by cells in 3D tissue. Co-expression of the inflammatory enzymes nitric oxide synthase 2 (Nos2) and cyclooxygenase 2 (Cox2) in triple negative breast cancer (TNBC) is indicative of an extremely poor prognosis. Nos2 and Cos2 interact in a proinflammatory feed-forward loop, mediated by Prostaglandin E2 (PGE2) and nitric oxide (NO). Nos2 produces NO, a key regulator of cancer processes, and is upregulated by hypoxia and nutrient deprivation. Here, we aim to: (1) determine how the spatial distribution of NO flux varies along the hypoxic gradient, and (2) determine how NO flux, mitochondrial mass, and mitochondrial activity change in response to Nos2 and Cox2 inhibition. To address these questions, we cultured 4T1 cells, a murine model of human TNBC, in the REEC system and quantified the cellular responses using live-cell and fluorescence microscopy. Inhibition of Nos2 resulted in a large compensatory increase in Nos2 expression in hypoxic regions, a drop in NO flux, as well as an increase in Cox2 expression primarily in normoxic regions. Mitochondrial activity was slightly elevated in hypoxic regions, and mitochondrial mass was decreased relative to controls. Inhibition of Cox2 abolished the spatial distribution of Nos2 and Cox2 observed in controls; Nos2 expression was elevated across the hypoxic gradient and Cox2 expression was elevated primarily in hypoxic regions. Mitochondrial activity was depressed and NO flux was very high. Our results support the conclusions that: (1) NO is a key regulator of mitochondrial biogenesis and mitochondrial activity, (2) Nos2 plays a key role in regulating the switch to anaerobic metabolism in hypoxic conditions, and (3) Cox2 plays a key role in regulating NO levels in response to varying levels of oxygen.

P799

**PCK2 balances TCA cycle flux and mitochondrial respiration to maintain the redox equilibrium in starved lung cancer cells**

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Cancer cells permanently face fluctuating nutrient levels and starvation conditions. To ensure survival and fast proliferation, cancer cells have to adapt their metabolism. Gluconeogenesis, in big parts the reverse pathway of glycolysis, has been recently described as survival strategy. Phosphoenolpyruvate carboxykinase (PEPCK, PCK) is the key gluconeogenic enzyme, catalyzing the conversion of oxaloacetate, a tricarboxylic acid (TCA) cycle intermediate, to phosphoenolpyruvate. The mitochondrial isoform of PEPCK, PCK2, is expressed in different tumor types, including lung cancer, and has been linked to pro-survival and pro-proliferative effects in vitro and in vivo. Here we show that silencing of PCK2 under glucose and serum starvation increases the amount of cellular TCA cycle intermediates in two different lung cancer cell lines, A549 and H23. The TCA cycle is connected to mitochondrial respiration via its production of reducing equivalents. Glucose and serum starvation led to increased mitochondrial respiration compared to non-starvation conditions, additionally PCK2 regulates oxidative phosphorylation in H23 and A549 lung cancer cells. Silencing of PCK2 resulted in an augmented basal oxygen consumption rate and maximal mitochondrial respiration whereas this effect was only minor in the case of non-starvation conditions. However, mitochondrial mass and mitochondrial structure remained mostly unchanged. In addition, PCK2 silencing provoked a small increase in mitochondrial superoxide levels. Notably, the amount of reduced glutathione (GSH), a key antioxidant defense molecule, was substantially decreased by PCK2 silencing. PCK2 silencing significantly reduced the colony forming ability of H23 and A549 cells, an effect partly abolished by addition of different antioxidants and enhanced by treatment with the oxidant H2O2. Key results, such as enhanced respiration, increased mitochondrial superoxide and reduced GSH/GSSG ratio could be mimicked through the addition of the TCA cycle intermediate dimethylmalate. In summary, we show that PCK2 plays a cataplerotic role in lung cancer cells, inhibiting excessive respiration and the formation of ROS under starvation conditions. Accordingly, PCK2 inhibition significantly impaired colony formation by starved lung cancer cells. In conclusion, PCK2 inhibition could potentially be utilized as a therapeutic approach to prevent metabolic adaptation and to enhance the formation of cell-damaging ROS in lung cancer cells.
vector systems were used. The levels of lipid and mitochondrial superoxide were assessed by MitoSOX and Bodipy stainings, respectively. Spheroid assay and transwell assay were used to evaluate migratory potential. Under glucose-deficient condition, the level of lipid was higher in TIL than in splenic T cells, and activated the lipid metabolism by upregulated SCD conferred phenotype of Th1 cells rather than those of Treg cells. Treatment of Jurkat cells with oleic acid upregulated SCD expression in glucose-deficient condition. Higher levels of IFN-γ, IL-2 and TNF-α were expressed in SCD-pLenti Jurkat cells than in parental and SCD-KO cells, while TGF-β was upregulated in the SCD-KO cells. Mitochondrial superoxide was increased in SCD-KO Jurkat cells more than in parental and SCD-pLenti cells, and quenching of the superoxide caused reduction of TGF-β and enhancement of IFN-γ, IL-2 and TNF-α. Additionally, SCD-pLenti Jurkat cells increased the migratory potential toward cancer cells through conjugation with ApoE. In contrast, SCD-KO cells showed lower migratory potential than parental cells. Consistently, splenic T cells showed identical pattern in the migratory potential and secretion of TGF-β, IFN-γ, IL-2 and TNF-α, following SCD regulation by inhibitor or inducer. Notably, overexpressed SCD in splenic T cells augmented the cancer-killing effect compared to the parental splenic T cells, but the cancer-killing effect was attenuated in splenic T cells by SCD inhibition. Our data illustrated that regulation of SCD expression in T cells could be potential way to enhance the efficacy of immune cancer therapy.

P801

Thioredoxin inhibits neutrophil-mediated proliferation of KRasG12V-transformed cells in zebrafish larvae

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Neutrophils in the tumor microenvironment have been shown to promote tumor growth and survival. Studies in zebrafish larvae using KRasG12V-transformed keratinocytes have demonstrated that neutrophils are instrumental in promoting transformed cell proliferation. Thus, the neutrophil response early in tumorigenesis likely promotes development and progression to cancer. To investigate the process of neutrophil recruitment to KRasG12V-transformed keratinocytes, we quantified gene expression via RNA sequencing and observed the antioxidant gene, thioredoxin (txn), to be upregulated in KRasG12V-expressing cells. Given the effects of reactive oxygen species (ROS) on innate immune cell recruitment and regulation, we sought to identify the role of thioredoxin in transformed cell fate and the innate immune response to KRasG12V-induced transformation using a txn+/− zebrafish line. We observed transformed cells in the txn+/− background were more frequently Edu-positive and displayed a reduction in active-caspase 3 staining, indicating an increase in proliferation and a decrease in apoptosis. Transformed cells in the txn+/− background also displayed increased tnfα expression, elevated oxidative redox status, and altered neutrophil dynamics suggesting an overall increase in the inflammatory status. Interestingly, ablating neutrophil recruitment to transformed cells via expression of dominant negative Rac2G57N within neutrophils restored transformed-cell Edu frequency to wildtype levels, indicating neutrophils are necessary for increased transformed cell proliferation in the txn+/− background. We therefore have found thioredoxin to be a tumor suppressor in early stages of KRasG12V-transformation, acting as an inhibitor to inflammation and subsequent neutrophil-mediated proliferation.
P802

Role if intracellular pH in cancer initiation, maintenance, and evolution
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Constitutively increased intracellular pH (pHi) and decreased extracellular pH (pHe) are emerging hallmarks of cancer found in most cancer types. This reversal of the pH gradient (pHi >7.4, pHe <7.2) is believed to be an early event in cancer development and has been shown to enable cancer-associated behaviors. In order to survive, cancer cells must adapt and respond to their ever-changing environment including this inverted pH gradient. However, it is unclear whether changes in pHi are sufficient contributors to cancer initiation, maintenance, or evolution. If increased pHi is a driver of cancer initiation, we hypothesized that early increases in pHi in a normal epithelial model could be sufficient to induce cancer-associated transcriptional changes. To address this question, we developed an experimental platform to transiently (24 hours) alter the pHi of normal human mammary epithelial cells (MCF10A). Our RNA-seq analysis revealed that experimentally increasing the pHi of MCF10A cells not only triggers a global change in transcriptional regulation, but also induces cancer-associated signaling and metabolic changes. This includes increased transcription of the signaling protein NOTCH1, which has been implicated in breast cancer progression, and increased transcription of lactate dehydrogenase (LDHA), which has been linked to cancer-associated Warburg metabolic reprogramming. Importantly, NOTCH1 and LDHA are significantly upregulated at the transcriptional level with increased pHi and significantly downregulated at low pHi, suggesting these nodes may act as pH sensors that can potentially regulate cancer-like behaviors in normal cells. We need better diagnostic markers of early cancer development or precancerous microenvironment pressures. Our results suggest that pHi (or associated molecular changes) could be used as diagnostic markers of early transformation and encourage development of pH-sensitive diagnostic tools. These diagnostic tools could additionally be used to identify patients that may respond to pH-lowering therapy.

P803

The pH-sensitive mechanism of IDH1-R132H, a recurrent somatic mutation in cancers
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An R132H mutation in the metabolic enzyme isocitrate dehydrogenase 1 (IDH1) is found in 70% of gliomas, and a further understanding of its functioning has the potential to lead to improved cancer diagnosis and treatment. IDH1 is important for regulating cytosolic levels of the key metabolite alpha-ketoglutarate as well as maintaining the reducing environment of the cell. While IDH1-WT converts isocitrate to alpha-ketoglutarate, IDH1-R132H converts alpha-ketoglutarate to the oncometabolite 2-hydroxyglutarate (2HG) in an oxidative reaction. Cancer cells have been shown to have an increased intracellular pH (pHi) compared to normal cells, and an inverted pH gradient. The IDH1-R132H mutation is the exchange of a basic amino acid, Arginine, with an amino acid, Histidine, with a pKa near neutral. We hypothesize that this charge-changing mutation allows IDH1-R132H to change its structure with
changes in the pH, functioning as a molecular switch to confer pH sensing. We found in cell-based assays that IDH1-R132H produces more 2HG at low pH, while IDH1-WT is pH-insensitive. Initial assays determined that neither IDH1-WT or IDH1-R132H exhibits pH-sensitive catalytic activity in vitro. However, prior work shows that 2HG production occurs fastest when both IDH1-R132H and IDH1-WT are present, suggesting that heterodimer formation is important for 2HG production. Thus we tested whether heterodimer formation is pH-sensitive and can confer the pH-sensitive 2HG production we observed in cell-based assays. We used in vitro native gel electrophoresis experiments to measure both homodimer and heterodimer formation at two different pH values. When IDH1-WT and IDH1-R132H were run on separate gels, IDH1-WT formed pH-insensitive homodimers while IDH1-R132H primarily remained as pH-insensitive monomers. We determined that low pH induced increased IDH1-WT and IDH1-R132H heterodimer formation. This suggests a mechanism by which pH-sensitive heterodimerization produces pH-sensitive catalytic activity and 2HG production. This data suggests that the IDH1-R132H mutant could be sensitive to cellular pH and cancer cell microenvironment conditions such as hypoxia, which has been shown to lower cellular pH. This pH-sensitive mechanism transforms our understanding of how this highly recurrent mutation functions, highlighting the complicated role of IDH1-R132H as a drug target and 2-hydroxyglutarate as a biomarker in gliomas.

P804

The nuclear Zn transporter ZIP11 is necessary for the proliferation of HeLa cells
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Zinc (Zn) is the second most abundant and essential trace element for all organisms. Under physiological conditions, Zn exists as a non-redox active divalent cation (Zn²⁺). Zn is part of several biological processes, including transcriptional regulation, signaling, catalysis, and as a structural component of proteins. A complex subcellular network of Zn transporters is indispensable to ensure the adequate distribution of Zn and to maintain homeostasis. Among these, the family of importers Zrt/Irt-like protein (ZIP) constitutes 14 members (ZIP1-ZIP14) that mobilize Zn into the cytosol. Some ZIP proteins can also transport iron, manganese, and cadmium. Research has shown that the expression of these transporters varies among tissues and during the different developmental stages. The presence of ZIP transporters at various cellular locations is essential for defining the net cellular transport of Zn. In cells, about 50% of Zn is cytosolic, 30-40% localizes in the nucleus, and 10% in the membrane. Normally, the ion is bound to proteins or sequestered in organelles and vesicles. Extensive research has focused on Zn internalization in mammalian cells. However, little attention has been given to the mobilization of the ion within the cells and the organelles, including the nucleus. In this regard, ZIP11 is the only ZIP transporter localized in the nucleus of mammalian cells. Analyses of HEK293T cells overexpressing Zip11 suggested that this transporter may mobilize Zn out of the nucleus. Sequence analyses indicated that Zip11 expression is responsive to Zn. However, the cellular role and the mechanism and direction of transport of ZIP11 are not defined yet. Therefore, we hypothesized that ZIP11 is a nuclear Zn transporter essential to maintaining nuclear Zn homeostasis in mammalian cells. To test this idea, our laboratory is using a well-established model of shRNA to knock-down Zip11 in normal fibroblasts and the cancer cell line HeLa. Preliminary data shows that partial deletion of Zip11 significantly reduced the proliferation rate of HeLa cells, and when HeLa cells reached confluency, they acquired an epithelial morphology. Experiments to determine the expression of epithelial vs. cancer markers are in progress. We will also investigate the
direction of transport of ZIP11 by measuring changes in nuclear and cytosolic Zn levels between wild type cells and cells with decreased expression of the transporter. Our work has the potential to discover a novel molecular mechanism where nuclear Zn homeostasis is an essential factor for cancer progression.

P805

**Suppressing ovarian cancer chemoresistance by targeting tumor microenvironment via SYK inhibition**

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The major barrier in improving the dismal outcome in advanced-stage ovarian cancer patients is the development of recurrent chemoresistant disease after primary debulking surgery and chemotherapy. There is an unmet need to test new therapeutic intervention to effectively manage ovarian cancer patients. To address this challenge, we set to investigate the molecular mechanisms underlying drug resistance and develop new therapeutics to enhance chemotherapy in ovarian carcinomas that are otherwise resistant. We found Spleen Tyrosine Kinase (SYK), commonly overexpressed in recurrent ovarian cancer, is the most promising target to revert drug resistance. The importance of SYK in non-hematopoietic cancer progression has only been recently recognized. We found that SYK is a promiscuous non-receptor kinase with multiple downstream substrates in ovarian cancer cells. Among the substrates are cortactin and coflin, two key regulators of actomyosin dynamics, both enhancing cell contractility and exosome exocytosis under the influence of SYK. We found that contractility is correlated with the cancer cells’ capacity of extracellular matrix (ECM) remodeling, which enhances the transportation of exosomes from cancer cells to stromal cells (e.g. fibroblasts, adipocytes, endothelial cells) and transformation of stromal cells into cancer-associated fibroblasts (CAFs), promoting tumor chemoresistance. We had established a Tetracycline-Off inducible mutant cell line to test whether SYK inhibition could suppress ECM remodeling and subsequent CAF induction. Indeed we observed SYK inhibition suppresses 50% of ECM remodeling by cancer spheroids. Moreover, SYK inhibition rescued normal human ovarian fibroblasts co-cultured with cancer cells from exhibiting CAF phenotypes. In summary, we demonstrate that SYK inhibition suppresses CAF induction by decreasing ECM remodeling and exosome secretion. Our result provides a biological foundation to apply the FDA-approved SYK inhibitor Fostamatinib (R406) to target tumor stroma as an adjuvant to currently available anti-cancer drugs.

P806

**Artesunate induces M1-like phenotypic changes of monocytes**

Introduction: In hematological malignancies, circulating monocytes are educated by tumor cells to assume an immunosuppressive M2-like phenotype and start to work in favor of tumor progression. Artemisinin (ARS) belongs to the established standard malaria treatments and presents also anticancer and immunomodulatory effects. Objective: Determine whether artesunate (ART), water-soluble derivative of ARS, is able to reverse the M2-like phenotype of monocytes. Methods: Blood samples were obtained from healthy donors and monocytes were isolated by gradient centrifugation. Control monocytes were cultured in RPMI 10% FBS and M2-phenotype was induced by IL-4 and then, M0 or M2-like monocytes were treated with increasing (100-500uM) doses of ART. Phenotypic changes were characterized through the expression of membrane markers and cytokine release, evaluated respectively by flow cytometry and ELISA, after 24h ART-treatment. Phosphorylation status of intracellular proteins were analyzed by Western Blot after 15 min ART-treatment. NHD13 mice, a transgenic animal that recapitulates features of the pre-leukemic disorder myelodysplasia (MDS), were treated intraperitoneally twice/week for 4 months with 100mgART/kg. Bone marrow and spleen samples were analyzed by flow cytometry. Results: Monocytes induced to a M2-like phenotype by Il4 and treated with ART presented an increased level of M1 markers such as HLA-DR expression (p=0.0043), MCP-1 and IL-1b release (p=0.0002 and p<0.0001, respectively). On the other hand, ART treatment reduced levels of mannose receptor CD206, a M2 marker (p<0.0001), in a dose-dependent manner. These findings were accompanied by decreased JAK2 and STAT3 phosphorylation. NHD13 mice treated with ART (n=4) showed increased percentage of CD45+CD11b+Gr-1high cells (p=0.0635) in bone marrows when compared with vehicle (n=5) treated mice. Conclusion: ART polarizes monocytes toward an inflammatory phenotype, suggesting a potential use in immunotherapy of cancer.

Tuesday, December 15, 2020, 1:00 pm

Actin Cytoskeleton in Immunology and Host-Pathogen Interaction

P807

Spatiotemporal oxidation of L-plastin downmodulates actin-dependent cellular functions

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Tumors possess a special redox homeostasis with a shift towards a prooxidative micromilieu. Moreover, several antitumor therapies work by increasing reactive oxygen species (ROS) within the tumor micromilieu. The potential influence of such therapies on immune cells required for antitumor response complicates the ROS influence on the tumor behavior. Therefore, studying regulation of redox sensitive proteins that are present in tumor cells and in immune cells would help to predict response to ROS inducing therapies. In our study, we revealed that L-plastin (LPL), an established tumor marker, was regulated by ROS-induced thiol oxidation on Cys101 and Cys42. LPL reduction was mediated by the Thioredoxin 1. LPL oxidation diminished its actin-bundling capacity which was accompanied by a marginal decrease in tumor cell migration, invasion and extracellular matrix degradation. Importantly,
focusing on spatiotemporal oxidation using ratiometric imaging of LPL-roGFP-Orp1 fusion protein and a
dimedone-based proximity ligation assay revealed that LPL oxidation occurs primarily in actin-based
cellular extrusions. Consequently, LPL spatial oxidation at the cell periphery strongly inhibited cell
spreading and formation of filopodial extension in tumor cells. Further investigation of spatiotemporal
oxidation of LPL in resting and stimulated T cells in response to several physiological stimuli pointed
towards involvement of spatial LPL oxidation on physiological functions of T cells as well as their
pathological suppression - depending on the amount and location of oxidized LPL. Altogether, these
findings highlight a delicate balance between the antioxidant capacity of cells, oxidation levels of LPL in a
spatial manner and actin-based cellular functions.

P808

Role of actomyosin complex and actin polymerization in Sendai virus assembly and egression
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Title: Role of actomyosin complex and actin polymerization in Sendai virus assembly and
egression
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Jana. ABSTRACT: Sendai virus (SV), a Murine Parainfluenza virus 1, has been widely used to study the
various stages of the Paramyxoviridae life cycle such as viral binding, fusion, replication, assembly and
subsequent egress. We have previously shown that Rho-ROCK- non muscle myosin II (NM II) contractility
signaling pathway may provide a physical barrier to host cells against viral fusion. But, whether the
actomyosin complex is involved during the viral assembly and egression is yet to be explored. Here, we
show that NM II and actin are associated with the viral particles only after the fusion step, and this
association reaches at maximum level at 24 hpi (the time point when the virus egress) as assessed by
ultracentrifugation at 1,00,000xg followed by immunoblot analysis when the Chinese hamster ovary
(CHO) cells are infected with SV. Such association with viral particles is shifted to 16 hpi when cells are
treated with Latrunculin A and Cytochalasin B, inhibitors of actin polymerization, as detected by
immunofluoroscence confocal microscopy and immunoblot analysis. Treatment with these actin
modulating drugs decrease the viral release in the culture media. All together, these data suggest that
both actin polymerization and association of NM II with the viral particles may modulate the viral
assembly and egression during the viral infection.

P809

Actin-binding proteins modulate the activity of actin-specific ADP-ribosyating toxins
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Bacterial pathogens developed multiple strategies to disrupt the actin cytoskeleton and compromise
cellular processes assisted by actin. Several ADP-ribosyating toxins (ARTs) from various pathogens
covalently modify one of two residues on actin - R177 or T148. ADP-ribosylation at R177 converts actin monomers (G-actin) into non-polymerizable species, leading to the cytoskeleton disruption. In contrast, modification of T148 results in abnormal actin dynamics and accumulation of F-actin aggregates. ADP-ribosylation by ARTs has classically been studied using G-actin as a substrate, however, under physiological conditions, G-actin is typically partnered with thymosin β4 (TMSB4) or profilin (PFN1). In this study, we evaluated the activity of three ARTs with physiologically relevant G-actin substrates. Among three R177-targeting ARTs, we found that PFN1-actin complex is a preferred substrate for *Salmonella enterica* ART SpvB, whereas *Aeromonas hydrophila* VgrG1 and *Clostridium perfringens* Iota toxins showed no substantial preference toward PFN1-actin or TMSB4-actin. Deletion of the poly-proline rich motifs from SpvB and VgrG1 toxins reduced but did not abolish PFN1’s effect on the efficiency of the enzymes. This observation confirms our hypothesis that the poly-proline rich motifs contribute to the binding of these ARTs to PFN1-actin complex, but also indicates that other elements contribute to the observed specificity. Latrunculin-B (LatB) caused similar potentiation effect of the SpvB’s ARTase activity as PFN1, suggesting that this effect can be mediated via a conformational change in the nucleotide cleft or the D-loop area on actin upon LatB binding that favors the catalytic modification of actin by SpvB. R177 modification caused no effect on the affinity of TMSB4 or PFN1 to G-actin. Given the recognized fast exchange of actin between TMSB4- and PFN1-bound pools, the substrate preference should not selectively affect PFN1-dependent or -independent actin polymerization pathways. Surprisingly, accumulation of R177-ADP-ribosylated-actin in cells inhibited the mDia1-controlled actin polymerization notably faster than the leading-edge retrograde flow of actin controlled by the Arp2/3 complex and myosin contractility. We speculate that the selectivity of SpvB towards the PFN1-actin pool may contribute to the selective toxicity towards different actin-dependent processes and reflect the need for intracellular pathogens to delicately regulate intracellular processes.

P810

**The role of actin and a novel interactor in *Giardia lamblia* attachment**

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*Giardia lamblia*, a protozoan parasite, is the cause of over 200 million annual cases of the debilitating gastrointestinal disease giardiasis worldwide. This extracellular parasite colonizes the lumen of the intestine, where it attaches to the microvilli in order to maintain infection. Attachment is mediated by the ventral disc, a specialized microtubule-based organelle. Since maintaining attachment is required for infection, modulation of ventral disc function is a promising area for new therapeutics, but it remains poorly understood. *Giardia lamblia* actin (glActin) is the most evolutionarily divergent actin described, with a 58% identity to the average actin, and lacks any conserved interactors. We recently identified a number of novel glActin interactors that localize to the ventral disc, suggesting an overlooked role for glActin in ventral disc function. One such protein, Disc and Actin-Related Protein (DARP), was particularly noteworthy because it appears highly enriched in the two regions of the disc important for fluid flow during attachment. DARP has no homologues or conserved identifiable protein domains. In this study, we investigated the role of both glActin and DARP in ventral disc function and morphology, particularly attachment. Using morpholino knockdown, we showed that glActin depletion results in decreased attachment to culture tubes and defects in disc morphology. Localization of DARP throughout the cell cycle revealed that it remains on the disc until late in mitosis. Using CRISPR-deadCas9 knockdown of DARP, we found a decreased ability of cells to remain attached to a surface, despite a
phenotypically normal disc. Our results indicate that both glActin and DARP are important for disc-mediated attachment and could be targets for novel therapeutics to treat giardiasis.

P811

**Bcl10 is associated with actin dynamics at the T cell immune synapse**

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T cell responses to antigen are initiated by engagement of the T cell receptor (TCR), leading to activation of diverse signaling cascades, including an incompletely defined pathway that triggers rapid remodeling of the actin cytoskeleton. Defects in control of actin dynamics and organization are associated with several human immunodeficiency diseases, emphasizing the importance of cytoskeletal remodeling in the functioning of the adaptive immune system. Here, we investigate the role of the adaptor protein Bcl10 in the control of actin dynamics. Although Bcl10 is primarily known as a component of the pathway connecting the TCR to activation of the NF-κB transcription factor, a few studies have implicated Bcl10 in antigen receptor-dependent control of actin polymerization and F-actin-dependent functional responses. However, the role of Bcl10 in regulation of cytoskeletal dynamics remains largely undefined. To investigate the contribution of Bcl10 in regulation of TCR-dependent cytoskeletal dynamics, we monitored actin dynamics at the immune synapse of primary murine CD8 effector T cells. Quantification of these dynamics reveals two distinct temporal phases distinguished by differences in speed and directionality. Our results indicate that effector CD8 T cells lacking Bcl10 display faster actin flows and more dynamic lamellipodia, compared to wild-type cells. These studies define a role for Bcl10 in TCR-dependent actin dynamics, emphasizing that Bcl10 has important cytoskeleton-directed functions that are likely independent of its role in transmission of NF-κB-activating signals.

P812

**An intrinsically disordered, glycine-rich protein governs mycobacterial actin-based motility**

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Examining how microbial pathogens manipulate the eukaryotic actin cytoskeleton during infection has yielded significant insight into the regulation of actin dynamics. Some intracellular pathogens co-opt the host actin polymerization machinery at the microbial surface to drive movement within and between host cells in a phenomenon known as actin-based motility. *Mycobacterium marinum*, a model organism to study the significant human pathogen *Mycobacterium tuberculosis*, stimulates actin-based motility within host cells by recruiting and stimulating the host proteins WASP and/or N-WASP to nucleate actin filaments through the Arp2/3 complex. However, the bacterial protein governing this phenomenon has remained elusive. Here, we report the identification of the *M. marinum* actin-based motility factor, which we designate mycobacterial intracellular rockets A (MirA). During infection, mirA mutant bacteria are unable to recruit WASP/N-WASP to stimulate actin polymerization at their surface, and are unable to spread from cell-to-cell. MirA co-localizes with N-WASP at sites of actin polymerization near or at the bacterial pole. Using an affinity purification/mass spectrometry approach, we show that MirA interacts with N-WASP along with N-WASP binding proteins CDC42 and the WIP family. Curiously, when expressed in eukaryotic cells, MirA also localizes to and stimulates actin polymerization at the surface of
host lipid droplet organelles. This propels lipid droplet organelles through the cytoplasm and raises the possibility that MirA may interact with host lipid droplets during infection for an unknown purpose. MirA is a member of the enigmatic PGRS protein family, the largest family of substrates translocated into host cells during mycobacterial infection. PGRS proteins are remarkably glycine-rich, and little is known of their functions due to their lack of homology to other proteins and sequence similarity with each other. Thus, MirA is radically dissimilar to known WASP/N-WASP binding partners and likely uses a distinctive mechanism for activating WASP/N-WASP. Further, dissecting the MirA-WASP interaction will glean new insights into actin regulation as well as reveal how the peculiar mycobacterial PGRS proteins are able to intercept host cell biological pathways.

P813

**CD13 Differentially Regulates the Formation of Cellular Protrusions**

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Distinct from filopodia, Tunneling Nanotubes (TNTs) are actin-based bridges suspended above the substratum linking two or more detached cells that exchange various cargoes. Whether filopodia and TNTs are indeed distinct entities or form via the same molecular mechanisms remains controversial. The transmembrane molecule, CD13, regulates Bradykinin-receptor signaling to promote filopodia formation and we have observed that TNT formation is diminished in cells lacking CD13. We explored the relationship between the formation of filopodia and TNTs by dissecting CD13’s role in each of these processes. Mechanistically, treatment of wildtype human endothelial cells with a CD13-activating antibody induced TNT, but reduced filopodia formation, in a CD13-dependent manner, suggesting that CD13-dependent signal transduction mediates TNT formation. Furthermore, while Bradykinin treatment potently induced filopodia, it had no affect on TNT formation, suggesting regulation by distinct signaling mechanisms. Interestingly, abundant accumulation of CD13 and its cytoskeletal binding partners at the base as well as along the length of both filopodia and TNTs may indicate that CD13 mediates membrane organization at the site of protrusion initiation. Our results suggest that CD13 plays a decisive and distinct role in both TNT and filopodia formation and future studies will elucidate potential differential mechanisms of formation.

P814

**Phagocytic adhesions and myosin II contribute in distinct ways to contractile forces that power phagocytic internalization**

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Phagocytosis is a remarkably versatile process that plays critical roles in the clearance of invading pathogens as well as the removal of dying cells in tissue homeostasis. Phagocytic engulfment of the target occurs through actin-polymerization mediated membrane extensions. How the actin cytoskeleton is reorganized and how the required forces are generated during target engulfment is, however, still largely unknown. Here, we combine rapid lattice light sheet microscopy (LLSM) imaging with
microparticle traction force microscopy (MP-TFM) for high-resolution quantification of actin dynamics and subcellular forces during phagocytosis. We show that phagocytic engulfment of soft IgG-opsonized hydrogel targets (~ 1 kPa) by macrophage-like RAW 264.7 cells involves strong - primarily contractile - target deformations that localize at the rim of the extending cup. The contractile ring bears striking actin-rich structures that locally protrude into the target (“teeth”). These teeth are highly dynamic and move over the target surface in coordinated fashion. Using fixed cells, we show that sites of target deformation precisely colocalize with areas of increased F-actin density, and that inhibition of Arp2/3-mediated branched actin polymerization abolishes both contractile forces and teeth formation throughout engulfment. In contrast, inhibition of myosin II using blebbistatin exclusively affects late stage force generation and contractile activity. Moreover, myosin II inhibition leads to an increase in the presence of late stage phagocytic cups (> 90% engulfment), suggesting a specific role of myosin II in late stage phagocytosis and cup closure. Observations of attempted partial target eating (trogocytosis) and of rapid resolving of conflicts when two phagocytic cells target a single particle, suggest that the contractile forces may help phagocytes to navigate and resolve some of the complex target encounters that can arise in vivo. Overall, our study reveals the presence of actin-polymerization-dependent protrusive forces and myosin II-dependent contractile forces that may contribute to mechanosensation and target engulfment during phagocytosis.

P815

A B cell actomyosin arc network couples integrin co-stimulation to mechanical force-dependent antigen centralization during B cell immune synapse formation

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B cells are the antibody-producing cells of the immune system that become activated when their B cell receptor (BCR) binds cognate antigen presented on the surface of an antigen presenting cell (APC). BCR: antigen interaction stimulates the formation of an immune synapse between the B cell and the APC that is driven by dramatic changes in the B cell cortical actin cytoskeleton. Importantly, ligation of the B cell integrin LFA-1 with ICAM-1 on the surface of the APC has been shown to promote B cell activation and immune synapse formation when membrane-bound antigen is limiting, as may often be the case in vivo. While this effect was attributed to an increase in B cell: APC adhesion, whether integrin co-stimulation also promotes B cell activation and synapse formation by altering the organization and dynamics of actin at the immune synapse remains unknown. Here we show using super-resolution imaging of primary B cells from myosin 2A-GFP knockin mice that LFA-1: ICAM-1 interaction in combination with BCR: antigen stimulation promotes the formation of a robust actomyosin network that colocalizes with integrin clusters. This network comprises the major actin network in the synapse, is created by the formin mDia1, and is organized into concentric, contractile arcs by bipolar filaments of myosin 2A. Quantitative time-lapse imaging showed that this network and the BCR: antigen clusters present within it flow inward at the same rate. Consistently, dynamic imaging revealed discrete BCR microclusters being swept inward by individual actomyosin arcs. Finally, inhibiting myosin contractility under conditions where integrin co-stimulation was required for immune synapse formation impaired this process, as evidenced by reduced antigen centralization, diminished BCR signaling, and altered CD19 distribution. Together, our results argue that a contractile actomyosin arc network created downstream of integrin co-stimulation plays an important role in the mechanism by which integrin co-stimulation promotes B cell activation.
Cell Death

P816

Characterizing pathways used by near infrared light to inhibit apoptosis
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Near infrared red (NIR) light exposure (2.88 J/cm², 632 nm) mediates cytoprotective effects in epithelial cells exposed to a pulse of 2 μm laser radiation. The overall goal of our research is to identify pathways by which NIR exposures inhibit apoptosis. Under ambient light and in normal cells, cytoprotection is mediated by NO-induced elevations in cGMP. Elevations in cGMP activate cGMP-dependent protein kinase I (PKG1) isozymes. PKG1 phosphorylates BCL2 - associated agonist of cell death (BAD), preventing it from binding BCL-XL, thus leading to decreased apoptosis. In order to determine if NIR light uses this pathway to mediate cytoprotection, retinal pigmented epithelial (RPE) cells were exposed to NIR light (2.88 J/cm², 632 nm). The cGMP ELISA by ENZO and the fluorescent molecule DAF-FM were used to quantify the levels of cGMP and NO within cells exposed to NIR light. RPE cells exposed to NIR showed increased NO (p< 0.05) and cGMP (p< 0.05) levels when compared to non-exposed cells. These data suggest that pathways that mediate apoptosis may be similar for ambient and NIR light. Future studies include using western blotting to examine downstream proteins in the apoptosis pathway.

P817

DDX3X scaffold function promotes prionoid phase transition and NLRP3 inflammasome activation
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Defects in stress granule machinery and aberrant NLRP3 inflammasome activation can promote inflammation in the brain. Stress granules can help the cell survive until the stressor is removed. Stress granules are membraneless compartments whose assembly requires liquid-liquid phase separation. NLRP3 inflammasome activation leads to the assembly of another cytoplasmic membraneless compartment called ASC specks and the induction of a form of pro-inflammatory programmed cell death called pyroptosis. ASC speck formation requires prionoid phase transitions. Thus, stress granules and NLRP3 inflammasomes involve radically different phase transitions and provide contrasting cell fate choices to a stressed cell - cell survival or pyroptosis. Cells utilize the limiting number of DDX3X molecules, the DDX3X cellular valency, to choose between the two cell fate choices. DDX3X is an integral component of stress granules. We have identified DDX3X as a novel regulator of NLRP3 inflammasome activation, making it a common essential factor for both stress granule assembly and NLRP3 inflammasome activation. Our data suggest that induction of stress granules inhibits NLRP3 inflammasome activation by limiting the available pool of DDX3X molecules, sequestering them in stress granules, thereby allowing the cell to make a pro-survival cell fate choice. These findings also suggest that stress granules and the NLRP3 inflammasome compete for DDX3X molecules, which makes DDX3X cellular valency critical for this decision-making process. Our data suggest that the ATPase activity of DDX3X is dispensable while its scaffold function is required for NLRP3 inflammasome activation. DDX3X-NLRP3 interactions are mediated by intrinsically disordered regions in the two proteins. Using an LPS-mediated peritonitis model, we further show that stress granules inhibit NLRP3 inflammasome activation in vivo. Our data point toward a mechanistic paradigm where cells exploit the cellular valency
of molecules and competition between biological processes for common essential factors to make cell fate decisions. **Funding Source:** T.-D.K. is supported by NIH grants AI101935, AI124346, AR056296 and CA163507 and by the American Lebanese Syrian Associated Charities

**P818**

**Round Up™ negatively impacts proliferation, cytoskeleton function, and calcium regulation to promote toxicity in chicken embryo finite cell line culture**

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The herbicide Round Up™ has become controversial in recent years due to reports of toxic effects in animals while manufacturers insist it is safe; its effects only target the plant specific shikimate pathway. Studies have recently challenged this view and a consequence have shown stunted cellular growth, reduced proliferation and apoptosis in animal cell cultures. Most toxicity findings focus on utilizing infinite cell lines, while easily accessible and widely available, do not provide an accurate representation of intrinsic cellular mechanisms due to abnormal ploidy and altered biochemistry. No studies to date have used whole animal cell in vitro cultures to assess toxicity of this widely used herbicide. By utilizing finite cell line cultures derived from chicken embryos, we could more closely mimic the normal milieu of an animals in an invitro environment. We therefore investigated Round Up™ toxicity using a chicken embryo-derived finite cell line as a near normal culture of cells for cellular toxicity. Our findings show a dramatic decrease in cellular viability with Round Up™ starting at concentrations 1,000 lower than domestic use formulations. After 2 days of culture in Round Up™ there was a 60% and 76% decrease in cell numbers in 10 ug/ml and 25 ug/ml Round Up™, respectively. Higher concentrations show even greater cell losses in the ranges of 79% and 93% for Round Up™ for 50 or 100 ug/ml. In 48 hours of culture we determined that cell growth occurred at a rate of 1,354 cells/hour, however in 10 ug/ml Round Up™, the cell loss rate was lower at ‐1,729 cells/hour and higher with greater concentrations of Round Up™. Interestingly, glyphosate alone did not produce the same toxicity at those specific concentrations. Furthermore, Round Up™ alterations of COS 7 cell culture of the cytoskeleton was monitored by microtubule immunofluorescence and show microtubule depolymerization. Intracellular Ca2+ levels were also altered and impaired under the treatment of Round Up™ which exhibit highly dynamic levels of this signaling molecule. Overall, we concluded that the formulation of Round Up™ exhibits greater toxicity than currently stated in most recent studies and formulations may contain adjuvants that are extremely toxic to animal cell cultures. This toxicity inhibits cellular proliferation, alters calcium homeostasis, and contributes to microtubule depolymerization. These changes have the potential to produce long-term negative consequences such as cancers or birth defects suggesting greater regulatory oversight on its use.

**P819**

**Metabolic determinants of cancer cell sensitivity to canonical ferroptosis inducers**

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Cancer cells rewire their metabolism and rely on endogenous antioxidants to mitigate lethal oxidative damage to lipids. However, the metabolic processes which modulate the response to lipid peroxidation are poorly defined. Using genetic screens, we compared metabolic genes essential for proliferation upon inhibition of cystine uptake or glutathione peroxidase-4 (GPX4). Interestingly, very few genes were commonly required under both conditions, suggesting that cystine limitation and GPX4 inhibition may impair proliferation via distinct mechanisms. Our screens also identify tetrahydrobiopterin (BH4) biosynthesis as an essential metabolic pathway upon GPX4 inhibition. Mechanistically, BH4 is a potent radical-trapping antioxidant that protects lipid membranes from autoxidation, alone and in synergy with Vitamin-E. Dihydrofolate reductase (DHFR) catalyzes the regeneration of BH4 and its inhibition by methotrexate synergizes with GPX4 inhibition. Altogether, our work identifies the mechanism by which BH4 acts as an endogenous antioxidant and provides a compendium of metabolic modifiers of lipid peroxidation.

P820

Vitronectin-mediated Cell Cycle Arrest Promotes Survival of MSCs under Different Physiological Stress Conditions.

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Mesenchymal stem cells (MSCs) are a candidate of choice for cell-based therapy and regenerative medicine owing to their immunomodulatory, anti-inflammatory, anti-apoptotic and homing properties executed via paracrine mechanism. However, harsh microenvironmental stress such as nutrient deprivation, hypoxia, inflammation etc at the target site post transplantation lead to massive cell death which is an obstacle towards realizing the full efficacy of MSC-based treatment. An in-depth understanding of MSCs survival response to such stress can help in redefining the strategies to improve the viability of MSCs post transplantation. Here we have evaluated the viability of umbilical cord-derived MSCs (UC-MSCs) and factors involved in the survival under serum deprivation and febrile temperature stress conditions. Under serum deprivation stress UC-MSCs adopted thin, elongated morphology with reduced proliferation and longer population doubling time. Further, the UC-MSCs underwent G0/G1 cell cycle arrest with no significant change in viability, when compared to control condition. Vitronectin (VTN), a multifunctional ECM glycoprotein, expression was strongly upregulated both at mRNA and protein levels. Immunofluorescence studies revealed changes in expression and distribution pattern of VTN between control and serum deprived UC-MSCs. VTN knockdown led to rescue in G0/G1 cell cycle arrest with significant decrease in viability suggesting its pro-survival role via promoting G0/G1 arrest. Next, signalling pathway analysis revealed that while NF-κB positively regulated, PI3K pathway negatively regulated VTN expression. Pro-survival PI3K pathway inhibition led to further increase in the VTN expression while cells continued to remain in G0/G1 arrest. Knockdown of VTN along with inhibition of PI3K pathway led to massive increase in apoptosis confirming the protective role of VTN under PI3K pathway inhibition state. Interestingly, febrile temperature stress, a hallmark of inflammation, also led to upregulation of VTN expression with change in morphology, G0/G1 cell cycle arrest and decrease in viability. Similar to serum starvation, VTN knockdown led to cell cycle arrest reversal with stronger decrease in viability. Overall, our study depicts the role and importance of VTN in prolonging survival of UC-MSCs under physiological stress conditions of serum deprivation and febrile temperature.
P821

**Caspase regulate the onset of extrusion through downregulation of an apical microtubule mesh**

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Cell extrusion is a sequence of remodelling steps leading to the removal of a cell from an epithelium without impairing its sealing. In previous work, the lab has shown that caspase activation precedes and is necessary for cell extrusion in the *Drosophila* pupal notum (a single layer epithelium). Thus we decided to study how effector caspases regulate the remodelling steps of cell extrusion in that tissue. So far, studies of cell extrusion mainly focused on the role and dynamics of actomyosin and E-cadherin, in agreement with their central role in the regulation of apical epithelial cell shape. We thus decided to conduct a quantitative phenomenology of these factors during extrusion in the notum. Surprisingly, the onset of constriction is not associated with a drastic change in apical MyoII levels or in apical actin dynamics. Moreover caspase inhibition in clones, which blocks cell extrusion, is associated with a significant increase in MyoII levels. Yet, MyoII is less effective for contracting cell apical area and does not lead to a clear increase of tension. Altogether this suggests that myosin accumulation is not limiting extrusion and that other factors regulated by caspases contribute to extrusion and apical constriction. Accordingly we found that the onset of cell apical constriction correlates systematically with the disappearance of an apical microtubule (MT) mesh (using different markers of MTs). Moreover, modulation of MT polymerisation led to a modulation of apical area. Furthermore, depleting MTs through colcemid injection can rescue extrusion in caspase inhibited cells. This suggests that the downregulation of this apical MT mesh by caspase is a rate limiting step for extrusion. More generally, this works outlines a so far neglected role of MTs in epithelial cell shape regulation.

P822

**Death of Escherichia coli during carbon starvation**

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Nutrient starvation is a defining part of the bacterial life cycle that usually leads to a decline in viability. The survival kinetics during the decline of viability are surprisingly simple - yet poorly understood. Non-sporulating bacteria like *Escherichia coli* show an exponential decay. In this talk, I will present our recent finding that death rate is set by just two factors, maintenance rate and recycling yield. Using this finding, I will show that bacteria adapt when growth slows down by reducing their maintenance rate. The ability to scavenge nutrients from carcasses of dead cells, the recycling yield, on the other hand, remains constant. As a result of the decreased maintenance rate, slow growing cells need less energy to survive than fast growing cells and die more than 5-fold slower. I will further show that the underlying cause for this adaptation is a fundamental trade-off between fast growth and long survival, set by the proteome allocation of the bacteria. This new and uncharacterized trade-off gives us a novel window on the mechanisms behind the spontaneous death of starving bacteria.
Caspase regulation under physiological stress
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Caspases are key players in determining cell fate and regulate pathophysiological processes affected by several environmental factors such as hyperosmotic, and cytotoxic stresses. Thus, caspases have been suggested as targets for the treatment of several diseases, including inflammatory and metabolic diseases. So far, most studies addressing caspases activation focused on acute rather than the physiologically-relevant gradual activation. Filling this knowledge gap will facilitate the understanding of the dynamic activation of caspases, which is essential to understand the mechanism of caspase activation in-vivo. To address this knowledge gap, we established a system to study the mechanisms of gradual caspase activation in cell culture mimicking in-vivo kinetics. Our preliminary data demonstrated that applying hyperosmotic stress gradually, rather than acutely, does not activate caspase signaling network, which in turn results in increased cell viability compared to acute stress. This demonstrates that differences between gradual and acute profiles. Thus, physiologically-relevant gradual administration of hyperosmotic stress is associated with significantly less activation of caspases as compared to acute administration.

Chemical Biology and Engineered Tissues

Hydrazine-based fluorescent probe for site-selective imaging of carbonylation in live cells
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Small-molecule fluorescent probes have been used for a wide variety of growing applications including bioconjugation, drug discovery, selective tagging of biomolecules in cells to be used for early diagnosis of several disease progression states such as cancer imaging. In order to introduce fluorescent probes into the living systems or preclinical applications, probes need to be designed on particular characteristics: having high stability, less toxicity, fast kinetics, good spectral properties (large Stokes shifts, reasonable quantum yields, drastic changes on absorption and emission spectra and minimal perturbation) are the essential key points to call them as a strong probe candidate in living systems. We designed and synthesized a hydrazine-based fluorescent probe which can react fast with the carbonyl moieties of biomolecules through a click reaction to produce a fluorescent product inside cells so that we can able to visualize carbonylation process in various cancer cell lines. Microscopic and spectrofluorometric analyses were used to differentiate the exogenous and endogenous ROS induced carbonylation profile in human dermal fibroblasts along with A498 primary site and ACHN metastatic site renal cell carcinoma (RRC) cell lines. Our results showed that our hydrazine-based fluorophore can selectively detect carbonylation level that differs in response to exogenous and endogenous stress in healthy and cancer cells. Use of bioconjugation chemistry method to monitor carbonylation with fluorescent probe has proven itself to be superior in satisfying many criteria (e.g., biocompatibility,
selectivity, yield, stability, and so forth); our results will therefore provide a powerful alternative to
conventional labeling strategies.

P825

**Wavy Morphology Mediates Vascular Smooth Muscle Cell Phenotype through Myosin**

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Vascular smooth muscle cells (VSMCs) reside in dense extracellular matrices that are organized in parallel and wavy bundles. The wavy structure, made of elastin and collagen fibers, contribute to the mechanical functionalities of elastic arteries, and can be lost in disease and aging. VSMCs follow this undulating organization and have wavy cell and nuclear morphology. To understand how this specialized structure regulates VSMC, we generated microchannels to study their phenotype. Cells conformed to the wavy structures with wavy actin cytoskeleton. Nuclei also conformed to the structures, exhibiting lateral compression from the actin stress fibers with increasing curvature. The wavy structure suppressed EdU incorporation, as compared with cells in straight channels. In addition, significantly higher immunofluorescence signal for α-smooth muscle actin (αSMA) was observed in the wavy patterns. These findings showed that the VSMCs exhibited a more contractile phenotype in the wavy structures, corresponding to a ‘healthier’ phenotype. To understand the intracellular signaling and structural mechanisms underlying this phenotypic regulation, we screened several cytoskeletal regulators using pharmacological treatments. Blebbistatin decreased αSMA organization and nuclear deformation and enhanced EdU incorporation in the wavy cells to levels similar to the straight cells. Using CK666, on the other hand, revealed that while Arp2/3 was involved in the maintenance of wavy cell morphology, it did not regulate morphology-mediated proliferation decline. These results indicate that myosin II-mediated cell contractility suppresses cell proliferation and potentially regulates additional phenotypic factors in the wavy cells. We previously reported increased cell tension, nuclear deformation, and chromatin organization in wavy fibroblasts. Furthermore, preliminary studies showed a role of chromatin organization in regulating nuclear shape in wavy VSMCs. Future studies will investigate additional cytoskeletal components and epigenetic regulation in these wavy cells. As VSMC phenotype is a significant mediator of vascular homeostasis, our study provides new insights in the regulation of VSMC phenotype and potential targets for treatments of cardiovascular diseases.

P826

**Trans-epithelial Fluid Pumping Performance of Renal Epithelial Cells and Mechanics of Cystic Expansion**

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Using a novel microfluidic platform to recapitulate fluid absorption activity of kidney cells, we report that renal epithelial cells can actively generate hydraulic pressure gradients across the epithelium. The fluidic flux declines with increasing hydraulic pressure until a stall pressure, at which the fluidic flux vanishes—in a manner similar to mechanical fluidic pumps. The developed pressure gradient translates
to a force of 50-100 nanoNewtons per cell. For normal human kidney cells, the fluidic flux is from apical to basal, and the pressure is higher on the basal side. For human polycystic kidney disease (PKD) cells, the fluidic flux is reversed from basal to apical with a significantly higher stall pressure. For mouse cells, complete deletion of PKD-2 gene decreased the apical-to-basal fluid flux but did not reverse it. Molecular studies and proteomic analysis reveal that renal epithelial cells are highly sensitive to hydraulic pressure gradients, developing different expression profiles and spatial arrangements of ion exchangers and the cytoskeleton in different pressure conditions. These results, together with data from osmotic and pharmacological perturbations of fluidic pumping, implicate mechanical force and hydraulic pressure as important variables during morphological changes in epithelial tubules, and provide further insights into pathophysiological mechanisms underlying the development of high luminal pressure within renal cysts.

**P827**

**Transcriptome Profile Alteration with Cadmium Selenide/Zinc Sulfide Quantum Dots in** *Saccharomyces cerevisiae*

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In our present study, we looked at the effects of yellow emitting CdSe/ZnS quantum dots (QDs) on the Baker’s Yeast *Saccharomyces cerevisiae*. We utilized experiments such as cell viability assays, RNA-seq, ROS detection assays, and cell wall integrity experiments to characterize the potential toxic effects of CdSe/ZnS QDs. We observed no negative effects on cell viability, but observed cell walls are more sensitive to CdSe/ZnS QD exposure than un-treated cells. Additionally, we observed no significant difference in the amount of superoxide in CdSe/ZnS treated cells compared to non-treated cells. According to our transcriptomic analysis, we found thousands of genes to be differentially expressed. Transcriptomic analysis allowed us to determine that exposure to CdSe/ZnS QDs significantly changed the gene expression in several cellular processes. Four significantly differentiated genes, including *FAF1*, *SDA1*, *DAN1*, and *TIR1* were used to validate our RNA-seq data by producing consistent gene expression results with RT-qPCR assays.

**Ciliopathies**

**P828**

**Conservation of the Genetic Interaction Between Bbs5 and Nphp4 Across Species**

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Primary cilia are microtubule-based structures that extend from nearly all mammalian cell types and play a role in cellular sensing and signal transduction. Ciliopathies are a spectrum of human disorders associated with defects in primary cilia formation and function. Ciliopathy patients present with a wide and variable range of clinical features, often with low genotype to phenotype correlation. It is believed that much of this phenotypic variability may stem from the presence of multiple modifier alleles causing
different degrees of primary cilia dysfunction. To identify these novel modifiers, we conducted an ethyl methanesulfonate (EMS) mutagenesis screen in *C. elegans* harboring a mutation in the ciliary transition zone component *nphp-4*. We aimed to identify secondary mutations that, when combined with an *nphp-4* mutation, caused defects in primary cilia formation or function. One such modifier mutation identified from this screen was mapped to the BBSome component *bbs-5*. The objective of this work is to test the evolutionary conservation of the genetic interaction between *bbs-5* and *nphp-4* identified in *C. elegans* in the vertebrate models, zebrafish and mouse. In *C. elegans*, we find that *bbs-5; nphp-4* double mutants display complete dye filling defects in ciliated sensory neurons. These animals also demonstrate abnormalities in cilia-mediated sensory functions including chemosensation and dauer formation. The abnormal sensory neuron function correlates with loss of proper ciliary compartmentalization and intraflagellar transport, but not with catastrophic ciliary structural defects. *Bbs5; Nphp4* zebrafish survive to adulthood at sub-Mendelian ratios. Those that reach adulthood display scoliosis and disorganization of the inner and outer segments of the retinal photoreceptor cells. The scoliotic and eye phenotypes are also observed with similar severity in adult *Bbs5* single mutant zebrafish. In contrast to either single mutant, mice harboring mutations in both *Bbs5* and *Nphp4* are embryonic lethal, with lethality occurring at either late embryonic or perinatal timepoints. Analysis of MEFs derived from embryonic *Bbs5*; *Nphp4* double mutants indicates that primary cilia are still present, suggesting that the embryonic lethality is caused by functional, but not structural ciliary defects. The lethality in zebrafish and mice indicate that as in *C. elegans* there are genetic interactions between *Nphp4* and *Bbs5* that exacerbate the phenotype. These combined results suggest that vertebrate and invertebrate models carrying mutations in both *Bbs5* and *Nphp4* display defects in primary cilia function and that these defects can have a significant effect on animal development and behavior.

P829

Ciliary ARL13B is critical to prevent obesity and diabetic phenotypes in mice
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*Tiffany Terry and Eduardo Gigante contributed equally to this project. ARL13b encodes a ciliary GTPase highly enriched in cilia. In mice, *Ar13b* null mutations are embryonic lethal with abnormal Sonic hedgehog (Shh) signaling believed to be due to their short cilia. To determine whether ARL13B is required in cilia for normal signaling, we generated a mouse line expressing a cilia-excluded ARL13B variant, *Arl13bV358A*. We found *Arl13bV358A/V358A* mice are viable and fertile, indicating ARL13B does not function from within cilia to control Shh signaling. However, we observed short cilia and improper trafficking of key ciliary proteins in *Arl13bV358A/V358A* cells. These results uncouple ARL13B function in cilia from Shh signaling regulation, demonstrating that ARL13B functions distinctly in cilia and the cell. Surprisingly, we found that *Arl13bV358A/V358A* mice gain significantly more weight than control littermates, suggesting that ARL13B function within cilia is critical for weight control. A connection between cilia and weight control is clear as some ciliopathies cause obesity due to ciliary dysfunction in neurons. However, the signaling mechanism within cilia that regulates hyperphagia-induced obesity is uncertain. The weight increases in *Arl13bV358A/V358A* mice began at five weeks of age, and by ten weeks of age, the mice were 33% heavier than controls. Since diabetes is often a comorbidity for obesity, we measured blood glucose changes in response to a single insulin or glucose dose. In a test of insulin tolerance, we found that *Arl13bV358A/V358A* mice are insulin resistant. In a glucose tolerance test, we measured high blood glucose levels that persist longer than in control mice. Taken together, these data argue that ARL13B plays
distinct roles within and outside of cilia and clearly implicates ciliary ARL13B as a critical regulator of feeding behavior.

P830

Ift-a deficiency in juvenile mice impairs biliary development and exacerbates adpkd liver disease

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Polycystic liver disease (PLD) is characterized by the growth of numerous biliary cysts and presents in patients with Autosomal Dominant Polycystic Kidney Disease (ADPKD), causing significant morbidity. Interestingly, deletion of intraflagellar transport-B (IFT-B) genes in adult mouse models of ADPKD attenuates severity of PKD and PLD. Here we examine the role of deletion of IFT-A gene, Thm1, in PLD of juvenile and adult Pkd2 conditional knock-out mice. Perinatal deletion of Thm1 results in disorganized and expanded biliary regions, biliary fibrosis, shortened primary cilia on CK19+ biliary epithelial cells, and reduced Notch signaling. In contrast, perinatal deletion of Pkd2 causes PLD, with multiple CK19+ biliary epithelial cell-lined cysts, fibrosis, lengthened primary cilia, and increased Notch and ERK signaling. Perinatal deletion of Thm1 in Pkd2 conditional knock-out mice increased hepatomegaly and liver necrosis, indicating enhanced liver disease severity. In contrast to effects in the developing liver, deletion of Thm1 in adult mice, alone and together with Pkd2, neither caused a biliary phenotype nor affected Pkd2-mutant PLD, respectively. However, similar to juvenile PLD, Notch and ERK signaling were increased in adult Pkd2-mutant cyst-lining cholangiocytes. Taken together, Thm1 is required for biliary tract development, likely by enabling Notch signaling, and proper biliary development restricts PLD severity. Unlike IFT-B genes, Thm1 does not affect hepatic cystogenesis, suggesting divergent regulation of signaling and cystogenic processes in the liver by IFT-B and -A. Notably, increased Notch signaling in cyst-lining cholangiocytes may indicate that aberrant activation of this pathway promotes hepatic cystogenesis, presenting as a novel potential therapeutic target.

P831

Shortened primary cilia in palatal mesenchyme of Spec1l<sup>ΔCCD2/ΔCCD2</sup> mouse model of cleft palate

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Orofacial clefts are among the most common congenital malformations, affecting approximately 1 in 700 births. Mutations in Spec1l have been identified in patients with syndromic and nonsyndromic cleft lip and/or palate. Syndromic Spec1l mutations cluster in the second coiled coil domain (CCD2), which facilitates interaction of this cytoskeletal protein with microtubules. Our recent studies indicate that mice with an in-frame deletion of Spec1l-CCD2 exhibit cleft palate and exencephaly in a dominant-negative manner. Notably, orofacial clefts and exencephaly are common manifestations of ciliopathies - human disorders which arise from disruption of cilia - although an association between Spec1l and cilia has not been reported. We have investigated cilia in E13.5 Spec1l<sup>ΔCCD2/ΔCCD2</sup> mutant palatal shelves. Spec1l localizes to the cytoplasm and around the cilia base in wild-type palatal mesenchyme, but this localization is diminished in Spec1l<sup>ΔCCD2/ΔCCD2</sup> palatal mesenchyme. In addition, primary cilia lengths are decreased in Spec1l<sup>ΔCCD2/ΔCCD2</sup> palatal mesenchyme relative to wild-type, on
both the lingual and buccal sides of the palate. We hypothesize that an intracellular trafficking defect in the palatal mesenchyme results in shortened cilia and that the shortened cilia affects signaling in the palatal mesenchyme. Since the IFT-A mouse mutant,,Thm1^{aln/aln}, shares overlapping phenotypes with Specc1l mutant mice, including cleft palate, exencephaly, and shortened cilia, we are crossing the two mutants to look for a genetic interaction. We are also investigating signaling molecules known to function in ciliogenesis and in palatal development.

Cytoskeleton in Membrane Fusion

P832

Small viral fusogens hijack distinct actin nucleation pathways to drive cell-cell fusion

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Cell-cell fusion is indispensable for numerous stages of development, from fertilization to tissue development and maintenance. It is typically driven by fusogenic membrane proteins that have tall (>10 nm) ectodomains that bridge the distance between plasma membranes. These tall fusogens often resemble those involved in enveloped viral entry and undergo conformational changes that pull the two membranes into close contact and drive fusion. In contrast, some cell-cell fusogens, such as the fusion-associated small transmembrane (FAST) proteins from reovirus, have ectodomains that are short (<2 nm), smaller than the repulsive hydration barrier that prevents membranes from coming into close contact. We previously found that one FAST protein, p14 from reptilian reovirus, hijacks the actin cytoskeleton to drive cell-cell fusion via the adaptor Grb2. However, the Grb2-binding motif identified in p14 is not found in any other FAST protein, suggesting that other FAST proteins either use alternate strategies to nucleate actin or use other cellular machineries to promote cell-cell fusion. Here, we report that an evolutionarily divergent FAST protein, p22 from aquareovirus, also hijacks the actin cytoskeleton but does so through different adaptor proteins. Using bioinformatics, chemical and genetic perturbations, fluorescence imaging, and molecular biological methods, we found that p22 binds to Intersectin-1 and subsequently Cdc42 to trigger N-WASP-nucleated branched actin assembly. We further show that despite arising from different gain-of-function evolution events, having minimal sequence similarity, and binding to different molecular players, the cytoplasmic tails of p22 and p14 can be exchanged to create a potent chimeric fusogen. This suggests that the extracellular and cytoplasmic domains of FAST proteins are modular and their specific molecular identity is secondary to a shared functional role. To further test this, we replaced p22’s branched actin nucleator, N-WASP, with the parallel actin filament nucleator, mDia2, a formin. Although filopodia nucleated by formins generate
parallel actin bundles different from space-filling branched actin networks, they can apply comparable mechanical pressures. Consistent with the idea that actin nucleation by these small fusogens generates mechanical force at the plasma membrane to bring the plasma membranes into close contact, we find that direct coupling of a fusion-null mutant to formin using an inducible dimerizer, FKBP-FRB, was sufficient to rescue cell-cell fusion. Altogether, this work points to a common biophysical strategy used by all FAST proteins to push rather than pull membranes together to drive fusion, one that may be harnessed by other short fusogens responsible for physiological cell-cell fusion.

P833

**Mechanism of cell-cell fusion induced by a bacterial pathogen**

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Cell-cell fusion is important for many biological processes including fertilization, development, bone maintenance, and immunity. Cell-cell fusion also contributes to microbial pathogenesis. For example, bacterial pathogens in the pseudomallei group of *Burkholderia* species, the causative agents of melioidosis and glanders, invade mammalian host cells, live in the cytosol, and spread directly from one host cell to another by inducing cell-cell fusion. The requirements for cell-cell fusion induced by one species in this group, *B. thailandensis*, include intracellular bacterial actin-based (or flagellar) motility and a bacterial protein secretion apparatus called the type VI secretion system. However, the temporal and spatial stages, as well as the molecular mechanisms of cell-cell fusion induced by *B. thailandensis* remain unknown. Using live cell imaging, we found that bacteria first use actin-based motility to push on the host cell plasma membrane to form protrusions that extend into neighboring cells. Then, fusion pores form within these membrane protrusions, either along their length (40%) or at the leading tip (60%). Finally, fusion pores expand to create a fused, multinucleated cell. Membrane protrusions formed by *B. thailandensis* likely help mediate membrane fusion by promoting the key molecular steps leading to fusion pore formation, including fusion protein localization and membrane apposition. In support of this notion, a *B. thailandensis* mutant deficient in a component of the type IV secretion system (VgrG5) enters into protrusions that get engulfed by neighboring cells rather than inducing cell-cell fusion. Interestingly, the *vgrg5* mutant cannot be functionally rescued by over-expression of VgrG5 in the host cell or by secretion of VgrG5 by neighboring wild type bacteria. This finding suggests that VgrG5 must be secreted locally by bacteria in protrusions to localize and/or function to promote fusion. Further study of cell-cell fusion induced by *Burkholderia* may lead to a better understanding of the basic mechanisms of cell-cell fusion in infected and uninfected cells.

P834

**Mechanoregulation of exocytosis rates by vesicle-membrane merging kinetics**

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Exocytosis is central to neurotransmission, hormone secretion and other fundamental processes. A common feature is that rates of exocytosis are up- or down-regulated when stimulus levels exceed a certain threshold. Neurons subject to high stimulus frequency exhibit facilitated or depressed synaptic strengths. Depression occurs in PC12 secretory cells (Gu et al, 2019). A well studied mechanism of depression is associated with depletion of the readily releasable pool (RRP) of release-competent...
vesicles. The actin cortex is also implicated as a regulator of exocytosis rates, serving as a physical barrier impeding secretory granule access to the plasma membrane (PM) (Vitale et al., 1995). The cortex is thought to regulate PM tension that promotes contents release (Bretou et al., 2014). Here we propose that an important mechanical factor regulating exocytosis rates is post-fusion vesicle-PM merging, whose kinetics limit release at high stimulus levels. A mathematical model of vesicle-PM merging kinetics shows that repeated delivery of secretory vesicles to a release site retards vesicle-PM merging and depresses release rates. The effect is mechanical: vesicle-PM merging lowers membrane tension locally, weakening the driving force for vesicle merger. We found that vesicle-PM merging is driven by mechanical factors: osmotic squeezing, membrane tension, and adhesion of the PM to the actin cortex (Shin et al, 2020). Osmotic squeezing abolishes vesicle membrane tension, creating a tension gradient that reels the vesicle into the PM, further assisted by membrane-actin cortex adhesion. A continuum model describes membrane tension dynamics and lipid flow, predicting vesicle merger times ~1 sec, agreeing with experiment (Shin et al, 2020). Importantly, membrane tension equilibrates slowly, hindered by adjacent actin cytoskeleton (Shi et al, 2018). Correspondingly, we identify a critical exocytosis rate above which membrane tension near a release site cannot recover, due to repeated vesicle merging events, each lowering the membrane tension due to the delivery of lipids. This dramatically lowers the driving force for PM-vesicle merger, and eventually prevents subsequently delivered vesicles merging with the PM and releasing their contents. This negative feedback depresses the exocytosis rate. The critical rate revealed by our model is comparable to the inverse time interval between repeated exocytosis events at hotspots recently identified in INS-1 cells (Yuan et al., 2015). We conclude that exocytosis rates may be regulated by mechanical effects associated with the actin cortex, through its effect on vesicle-PM merging, and that exocytic depression previously attributed to RRP depletion may originate in part from these ubiquitous biophysical effects.

Endosomes

P835

Glucose regulated trafficking of cell surface membrane proteins

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Glucose regulated trafficking of cell surface membrane proteins. Eukaryotic cells adapt their metabolism to the extracellular environment. Downregulation of cell surface cargo proteins in response to nutrient stress reduces the burden of anabolic processes whilst elevating catabolic production in the lysosome. We use yeast genetics, quantitative gene expression profiling, 4D confocal microscopy, and biochemical approaches to show that glucose starvation triggers a transcriptional response that simultaneously increases internalisation from the plasma membrane whilst suppressing recycling from endosomes back to the surface. Nuclear export of the Mig1 transcriptional repressor in response to glucose starvation increases expression of 1) the yeast AP180 clathrin adaptors (YAP1801 / YAP1802), which is sufficient to increase cargo internalisation, and 2) the G-alpha subunit GPA2, which antagonises endosomal recycling mediated by the associated yeast phosphatidylinositol 3-kinase regulator, Gpa1. We also show glucose starvation results in Mig1-independent transcriptional upregulation of many eisosomal factors, which serve to sequester a portion of nutrient transporter cargoes to persist the starvation period and maximise nutrient uptake upon return to replete conditions. The eisosomal factor Pil1, which we find is rapidly dephosphorylated following glucose starvation, undergoes significant
conformational changes specifically at eisosomes that retain cargo, with marked changes in Pil1 stoichiometry and diffusion coefficients observed with single molecule biophysical approaches. We propose the uncharacterised eisosomal protein Ygr130c acts as a physical cargo retention factor, to anchor nutrient transporters to eisosomes. Furthermore, we show this cargo retention mechanism provides a physiological benefit for cells to rapidly recover from glucose starvation. Collectively, this remodelling of the surface protein landscape during glucose starvation calibrates metabolism to available nutrients.

P836

Role of iron transporter DMT1 in endosome-mitochondria interaction in breast cancer cells
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In tumoral cells iron is essential to support higher cell proliferation as well as stabilization of metalloproteases, influencing extracellular matrix degradation and metastasis. Recently, we had shown that interaction between early endosomes (EE) and mitochondria regulates intracellular iron transport in epithelial cells. Since mitochondria are the ultimate cellular iron sink, it is important to understand how endosome-mitochondria interactions are regulated to modulate iron transport in tumoral cells. Here, we had start to evaluate the role of the iron transporter DMT1 in EE-mitochondria interaction, iron transport into mitochondria and its functional influence in proliferation and invasion. Leica Thunder microscope and LAS software were used for the imaging of MDA-MB-231 cells grown in 2D cell culture and 3D tumor spheroid systems. Higher resolution imaging was performed using Airyscan LSM880. Z-stack images were subjected to 3D rendering using IMARIS software to evaluate organelle morphology and organelle-organelle interactions. For silencing experiments, we used CRISPR/Cas9 validated by immunoblotting. Expression of different proteins related to iron transport and signaling, e.g. DMT1, mitochondrial-ferritin, transferrin receptor and EGFR showed substantial heterogeneity between non-cancerous epithelial cells MCF10A and breast cancer cells MDA-MB-231 and T47D. DMT1 knock-out (KO) in both MDA-MB-231 and T47D decreases ERK and AKT activation and also cell proliferation and invasion. MDA-MB-231 DMT1 KO and T47D DMT1 KO shows alterations in EE and late endosomes size. DMT1 also appears to have a role in the association between mitochondria with both EE and late endosomes. In 3D culture of MDA-MB-231 tumor spheroids, we observed significant heterogeneity in EE and mitochondria morphologies, suggesting that 3D cancer cell growth could influence EE-mitochondria interactions and iron transport. Overall, our results suggest that iron transporter DMT1 can regulate EE endosomal size and EE-mitochondria interactions required for iron import into mitochondria and may be at the basis of more aggressive tumor phenotypes in breast cancer.

P837

Rabip4' interacts with Arl8b and mediate retrograde trafficking from endosomes to trans-Golgi Network
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Lysosomes are primarily known for their catabolic function in degradation of cellular macromolecules, but recent findings reveal that lysosome is also a site for processes like nutrient signaling and plasma membrane repair and in the long-range transport of RNA. Lysosomal sub-cellular distribution and fusion with other compartments govern its diverse roles in the cell. Our research group is interested in small GTP-binding protein, Arl8b and its effectors in regulating lysosomal positioning and cargo trafficking to lysosomes. Arl8b localizes on lysosomes and recruits effector proteins that mediates its motility and fusion with other endocytic compartments. Here we report a new Arl8b interaction partner, Rabip4 that interacts with Arl8b via its N-terminal RUN domain. We find that Rabip4 and Arl8b colocalize on a subset of early endocytic compartments that also contain Rab14 and the Cation-Independent Mannose-6 phosphate receptor (CI-M6PR). Arl8b siRNA treated cells show redistribution of Rabip4 from its normal punctate distribution to the cytosol, suggesting that Arl8b is required for stable membrane localization of Rabip4. To investigate the role of Rabip4 in regulating trafficking and function of late endocytic compartments, we visualized late endosomes/lysosomes in Rabip4 siRNA treated cells. Depletion of Rabip4 leads to increase in the size of LAMP1-positive compartment and enhanced accumulation of lysotracker dye that is retained only in acidic organelles. Also, cargo degradation by lysosomes in Rabip4 depletion is less efficient, as assessed by DQ-BSA assay. Rabip4 siRNA treatment in HeLa cells leads to secretion of the pro forms of lysosomal aspartic protease cathepsin D, a defect of cargo missorting from Golgi causing lysosome dysfunction. Lysosomal hydrolases are sorted in CI-M6PR-dependent manner and defect in recycling of CI-M6PR receptor causes enlargement of endo-lysosomal compartments. Using CD8-M6PR trafficking assay, we demonstrate that Rabip4 compartment regulates the CI-M6PR retrieval to trans-Golgi network (TGN) and its depletion leads to retention of CI-M6PR in endosomes. In summary, we have identified that Rabip4 interacts with Arl8b on a subpopulation of endosomes containing Rab14. Furthermore, by regulating cargo trafficking of CI-M6PR from endosomes-to-TGN, Rabip4 regulates lysosome composition and function.

P838

Inhibition of PI3K disturbs heterotypic endosome fusion
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[Introduction] Endocytosis is a cellular process that involves membrane-derived vesicle for recycling of intra- and extracellular components. Autophagy is also related to the cytosolic membrane vesicles called autophagosome and plays an essential role in degrading the dysfunctional proteins or organelles. Both endocytosis and autophagy have a common endpoint at the lysosome, which acts as a waste disposable system, and concertedly contribute to maintain intracellular homeostasis. The functions of the endocytosis and autophagy are well studied but its mechanisms such as the vesicle formation, fusion and trafficking are poorly understood. Phosphoinositide 3-kinases (PI3Ks) are well known regulator of membrane traffic through the endocytosis and autophagy. The class III PI3K controls endosome maturation and is also involved in the early steps of autophagic pathway such as the formation of the autophagosomesal-sequestering vesicle. In contrast with the early phase of vesicle formation, the role of PI3K on later pathway such as a heterotypic endosome fusion with lysosome or autophagosome remains unclear. In this study, we investigate the role of PI3K by using fusion model of heterotypic vesicles with live-cell imaging technology.[Results] ECGreen, a small fluorescent lipophilic dye, is internalized via endocytosis and its fluorescence intensity is enhanced according to acidity increase. Having these characteristics, ECGreen allows a direct observation of the dynamics of the endocytic vesicles in live cell.
We have applied ECGreen to investigate the effect of PI3K inhibition on a heterotypic endosome fusion. Our co-localization studies revealed that ECGreen was co-located with red fluorescent protein (RFP) - tagged with Rab5 and LAMP1 (early endosome and lysosome marker) in HeLa cells, showing ECGreen stains endocytic vesicles. In addition, ECGreen-stained endocytic vesicles were co-located with RFP-LC3 (autophagosome marker) under the starvation. In the presence of wortmannin (PI3K inhibitor), Rab5-positive vacuoles were imaged as ring-shaped structures, indicating the swollen endosome phenotype. ECGreen-stained endocytic vesicles also exhibited swollen endosome phenotype by wortmannin treatment and its ring structures were co-localized with Rab5, but not co-localized with LAMP1. Moreover, ECGreen-stained swollen endosome was LC3-negative even if under the starvation.

[Conclusion] In this study, we highlighted the role of PI3K on later pathway of endocytosis and autophagy, i.e., fusion of heterotypic vesicles. Our live-cell imaging with ECGreen revealed that endosome do not fuse lysosome or autophagosome when PI3K is inhibited, suggesting that PI3K could play a pivotal role in late phase of endocytic and autophagic pathway.

P839

De novo macromolecular peptides for inhibiting, stabilising and probing the function of the Retromer endosomal trafficking complex

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Maintenance of appropriate levels of endocytic trafficking and subsequent sorting in endosomes is essential for every aspect of cellular life. The evolutionarily conserved Retromer complex (composed of VPS35-VPS26-VPS29) and associated sorting nexin (SNX) proteins are central mediators responsible for this processing in endosomal compartments in all eukaryotes. It is known that mutations in Retromer can cause late-onset Parkinson’s disease, and can also be hijacked by viral and bacterial pathogens during cellular infection. Seeking tools to modulate Retromer function would provide new avenues in understanding Retromer function and the associated diseases. Here we employed the random nonstandard peptides integrated discovery (RaPID) approach to identify a group of cyclic peptides capable of binding to the Retromer complex with high affinity and specificity. Five of cyclic peptides bind to Vps29 via a di-peptide Pro-Leu sequence. Our structures show that these peptides structurally mimic known interacting proteins including TBC1D5, VARP, and the bacterial effector RidL, and potently inhibit their interaction with Retromer in vitro and in cells. Further analysis showed that a unique cyclic peptide binds Retromer at the interface between Vps35 and Vps26 subunits andshowing minimal disruptive effects on Retromer’s ability to associate with known accessory proteins and cargo adaptors in vitro and in cellular lysates, which makes it an ideal molecular chaperone to stabilise the Retromer complex. Finally, we show that these peptides can also be used as tools for probing Retromer function. Using reversible cell permeabilization and fluorescent peptides, we demonstrate that both the Retromer inhibitors and stabilizers can specifically co-label Vps35-positive endosomal structures, and can be used as baits for purifying Retromer from cells and subsequent proteomic analyses. We believe these cyclic peptides can be used as a toolbox for the study of Retromer-mediated endosomal trafficking, and sheds light on developing novel therapeutic modifiers of Retromer function.
Bro1 directly stimulates Vps4 activity to promote Intralumenal Vesicle Formation during Multivesicular Body biogenesis in yeast

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Endosomal sorting complexes required for transport (ESCRT-0, -I, -II, -III) participate in membrane remodeling events including intraluminal vesicle (ILV) formation during multivesicular body (MVB) biogenesis, viral budding, cytokinesis, autophagy, membrane damage repair, and extracellular vesicle biogenesis. During MVB biogenesis, early ESCRTs (–0, –I, –II) recognize ubiquitin (Ub)-modified cargo and sequester them for incorporation into ILVs that are generated by the action of ESCRT-III and associated factors Vps4 and Bro1. Ub modification serves as a signal for cargo selection by Ub-binding domains within early ESCRTs, and previous work has revealed a requirement for Ub-cargo for ILV formation to occur. How cargo sorting and ILV formation are coordinated remains unclear. Bro1, the yeast homolog of mammalian ALIX and HD-PTP, promotes ESCRT-III assembly by impeding AAA-ATPase Vps4 mediated ESCRT-III disassembly. In addition, Bro1 also participates in Ub-modified protein cargo sorting into MVBs as a Ub receptor. Here we demonstrate the evolutionarily conserved “V domains” from Bro1-family proteins directly stimulate Vps4 ATPase activity. This mode of stimulation is physiologically relevant as mutants defective for Vps4 stimulation display MVB sorting defects \textit{in vivo}. Additional analyses suggest this mode of Vps4 stimulation is relevant for the expansion phase of ILV biogenesis. Surprisingly, a carboxyl-terminal fragment containing the V domain of Bro1 supports ILV formation but not sorting of protein cargo into ILVs, revealing that cargo sorting and ILV formation can be uncoupled. Moreover, Ub binding by the V domain promotes this ILV formation independent of protein cargo sorting \textit{in vivo} and enhances V domain stimulation of Vps4 \textit{in vitro}. These studies indicate: 1) Ub can modulate ESCRT-III-dependent ILV formation through Bro1 and Vps4 in addition to its previously appreciated role in cargo recognition, 2) Vps4 activity contributes to ILV biogenesis prior to scission, and 3) Bro1 may be a “licensing” factor coordinating cargo sorting with direct regulation of Vps4 to modulate ESCRT-III driven ILV formation during MVB biogenesis.

Elevating PI3P drives select downstream membrane trafficking pathways

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Phosphoinositide signaling lipids are essential for several cellular processes. The requirement for a phosphoinositide is generally established by depletion of the corresponding lipid kinase. However, there are few reports on the impact of elevating phosphoinositides. That phosphoinositides are dynamically...
Elevated in response to stimuli suggests that in addition to being required, phosphoinositides drive downstream pathways. To test this hypothesis, we elevated the levels of phosphatidylinositol 3-phosphate (PI3P) by generating hyperactive alleles of the yeast phosphatidylinositol 3-kinase, Vps34. We find that hyperactive Vps34 drives select pathways, including PI(3,5)P2 synthesis and retrograde transport from the vacuole and endosomes, demonstrating that PI3P can be rate limiting in some pathways. Interestingly, hyperactive Vps34 does not affect ESCRT function at endosomes or the vacuole. Thus, driving a PI3P-dependent step does not always increase the overall rate of a complex pathway. Elevating PI3P can also delay a pathway. Elevating PI3P slowed late steps in autophagy, in part by delaying the disassembly of autophagy proteins from mature autophagosomes as well as delaying fusion of autophagosomes with the vacuole. Overall, these studies suggest that stimulus-induced elevation of phosphoinositides provides a way for stimuli to selectively regulate pathways.

P842

Deficiency in the Endosomal Sorting Factor HD-PTP Perturbs Cell Signaling
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Endosomal Sorting Complexes Required for Transport (ESCRTs) recognize ubiquitin-modified cargoes and incorporate them into intraluminal vesicles (ILVs) during multivesicular body (MVB) formation. Heterotypic fusion of MVBs with the lysosome results in degradation of the ILVs and their contents; this sorting event impacts cellular physiology by terminating signaling through activated receptors and modulating cell surface protein composition. Bro1 Family Proteins - including Bro1 in yeast and ALIX and HD-PTP in mammalian systems - contribute to both cargo recognition and membrane deformation during ESCRT-mediated processes. ALIX has been implicated in functioning with ESCRTs in viral budding, cytokinesis, and exosome biogenesis; in contrast, HD-PTP has been primarily implicated in MVB sorting for receptor degradation. Previous studies on the Drosophila HD-PTP homolog, Myopic, have suggested that HD-PTP is additionally involved in promoting signaling through Epidermal Growth Factor Receptor (EGFR) and Toll receptor. Reverse Phase Protein Array (RPPA) and RNAseq analysis of tissue from hypomorphic HD-PTP (HD-PTPh/h) mice revealed alterations in PI3K/AKT, RAS/MAPK, and NFkB signaling pathways. While RPPA suggests HD-PTP exhibits a similar function in murine cells by promoting activation, these effects could also result from altered endocrine signaling rather than via a cell autonomous mode. To address this issue, in vitro LPS stimulation of HD-PTPh/h peritoneal macrophages was performed and revealed perturbed NFkB signaling, indicating cell autonomous mechanisms contribute to the observed alterations. Similarly, an inducible HD-PTP knock-out MEF cell line exhibited reductions in both EGF induced ERK activation and LPS induced IKK activation. These observations suggest that Myopic’s role in promoting receptor signaling in Drosophila is conserved for HD-PTP function in mammalian EGF and Toll-like Receptor signaling pathways.

P843

Regulatory Function of GCN5L1 In Lysosomal Homeostasis and Biogenesis
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Lysosomes are responsible for degradation of macromolecules through fusion with autophagosomes and endosomes. The consumed lysosomes are required to be regenerated through the process of
autolysosomal reformation (ALR) and lysosome biogenesis. This project aimed to investigate the regulatory mechanism of GCN5L1 gene in lysosome tubulation (LT), a crucial step for ALR. GCN5L1 belongs to both BLOC1 and BORC multiprotein complexes and is involved in controlling lysosomal trafficking, however, the effect of GCN5L1 on lysosome tubulation remains largely unknown. Genetic ablation of GCN5L1 in the liver showed a dramatic increase in accumulation of autolysosomes, decrease in lysosome regeneration and absence of lysosomal tubulation. This phenotype suggests the possibility of disruption in lysosome tubulation which results in disturbance of the overall lysosome homeostasis. Interestingly, the genetic reintroduction of GCN5L1 rescues LT in GCN5L1 knockout hepatocytes. To investigate if GCN5L1 regulates lysosomal components, Lyso-IP method was used to purify lysosomes from WT and GCN5L1-/- hepatocytes followed by quantification of lysosomal protein expression profiles using label free LC-MS. Bioinformatic analysis revealed that expression of lysosomal protease, Cathepsin B, was reduced while there was a significant increase in autophagy-related proteins in GCN5L1-/- hepatocytes. These results support that the autophagic flux may be blocked due to inefficient digestion of lysosomes which results in accumulation of autolysosomes in GCN5L1-/- hepatocytes. Consequently, the autolysosome-dependent mTORC1 activity was upregulated in GCN5L1-/- hepatocytes. Our findings suggest that GCN5L1 deficiency alters the lysosomal signaling and contents as well as their degradation. These findings support that GCN5L1 plays a novel regulatory role in lysosome biology and may be involved in pathological processes of lysosome related disease, such as storage diseases, hepatic and cardiovascular diseases as well as the immune system function.

P844

**Bloc-1 plays a role in planar cell polarity and vangl2 trafficking**

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Planar cell polarity (PCP) is crucial during development, and is the coordinated cellular polarity along an axis parallel to the plane of the tissue. Defects in PCP have been linked to human pathology such as neural tube defects. Furthermore, these defects result in mis-orientation of hair cell stereociliary bundles in the epithelium of the cochlea and deafness. One defining feature of PCP is the asymmetrical localization of core PCP proteins, Vangl2 and Frizzled. One long standing question in the field is what is the cellular machinery that regulates the sorting of PCP transmembrane proteins. In this study, we examine the role of biogenesis of lysosome-related organelles complex-1 (BLOC-1) in PCP and Vangl2 trafficking. First we evaluated the localization of Vangl2 in cochlea and cristae from loss of function BLOC-1 mice, Dtnbp1<sup>sdv/sdv</sup> and Pldn<sup>pa/pa</sup>. There was a reduced membrane localization of Vangl2 in Pldn<sup>pa/pa</sup> mice. We observed notable PCP defects including mis-oriented hair cell stereociliary bundles and extra hair cell rows in Pldn<sup>pa/pa</sup> mice. These finding suggest that BLOC-1 mediated endosomal pathways is required for PCP sorting and membrane targeting of Vangl2.
Establishing and Maintaining Organelle Structure: Organelle Interaction and Tethering

P845

**Motion of single molecular tethers reveals dynamic subdomains at organelle contact sites**

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Membrane-bound organelles provide distinct compartments where incompatible biological processes can be separated from one another. However, biochemical reactions in these disparate environments must be coordinated to facilitate homeostasis, particularly in response to environmental changes. Sites of direct contact between the organelles play an important role in this process, serving as signaling hubs and locations of direct transfer for macromolecules. Mechanisms for regulating contact site structure and function remain enigmatic, but it seems likely to involve substantial heterogeneity and plasticity within the system. Numerous specific molecular tethers have been implicated in the process, but the small size and dynamic nature of contact sites have prevented understanding of their spatiotemporal regulation. Here, we use high-speed single molecule imaging to directly observe tethering and release dynamics of putative tethers in individual contact sites between the ER and mitochondria. We demonstrate the existence of structurally regulated subdomains within single contact sites by comparative imaging with 3D electron microscopy of vitreously-frozen samples. We show that distinct tethering molecules have unique patterns of motion, respond differently to nutrient availability, and can actively exclude one another from regions of high density. Thus, changes in the recruitment efficiency of individual tethers can support a change in contact site function without necessitating the breaking of one contact site and formation of another.

P846

**Loss of mitochondria-plasma membrane tethering adversely impacts organelle function and cellular fitness**

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To ensure cell survival, the processes that maintain cellular homeostasis and fitness must be coordinated and tightly regulated. To coordinate these processes, the membrane-bound organelles that compartmentalize distinct cellular activities must communicate with each other or the plasma membrane (PM). This communication is facilitated by membrane contact sites (MCSs), which position organelles in close proximity without membrane fusion and mark sites of critical activities necessary for proper organelle function, such as the exchange of molecules. Thus, the proper positioning of organelles and the contacts they make are deeply connected to the ability of these organelles to engage in their essential functions. Despite their importance to cellular fitness, the molecular mechanisms and regulation of MCSs remain unclear. Our research seeks to gain insight into the molecular mechanisms that allow MCSs to integrate critical cellular functions through the study of Num1, the core component of the Mitochondria-ER-Cortex-Anchor (MECA). Num1 has dual functions: (1) tethering mitochondria to the PM and (2) tethering the dynein motor protein to the PM during nuclear inheritance. Our research suggests that Num1 serves as more than a physical tether for mitochondria but is important for
maintaining proper functioning mitochondria. Specifically, loss of Num1 results in a growth defect when cells are forced to respire, which requires functional mitochondria. Consistent with a defect in mitochondrial function basal oxygen consumption is reduced. These mitochondrial defects are not due specifically to the loss of mitochondrial morphology or defects in the formation of respiratory complexes, and the growth defect is separate from Num1’s role in nuclear migration. Using a synthetic mitochondria-PM tethering system, our data demonstrate that mitochondrial function can only be rescued by the membrane binding domains of S. cerevisiae Num1 and no other mitochondrial or PM binding domains. These data suggest Num1 does more than simply position mitochondria; instead Num1’s membrane binding domains form a unique functional interface between mitochondria and the PM that maintains mitochondrial and cellular function.

P847

Phosphorylation switches on Endoplasmic Reticulum-Organelle contacts
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Organelles are physically connected in membrane contact sites. The endoplasmic reticulum possesses three major receptors, VAP-A, VAP-B and MOSPD2, which interact with proteins at the surface of other organelles to build contacts. VAP-A, VAP-B and MOSPD2 contain an MSP domain which binds a motif named FFAT [two phenylalanines in an acidic tract]. In this study, we identified a non-conventional FFAT motif where a conserved acidic residue is replaced by a serine/threonine. We show that phosphorylation of this serine/threonine is critical for non-conventional FFAT motifs (named Phospho-FFAT) to be recognized by the MSP domain. Moreover, structural analyses of the MSP domain alone or in complex with conventional and Phospho-FFAT peptides revealed new mechanisms of interaction. Using a prototypical tethering complex made by STARD3 and VAP, we showed that phosphorylation is instrumental for the formation of ER-endosome contacts, and their sterol transfer function. Based on these new insights, we produced a novel prediction algorithm which expands the repertoire of candidate proteins with a Phospho-FFAT able to create membrane contact sites. This study reveals that phosphorylation acts as a general switch for inter-organelle contacts.

P848

Drp-1 deficiency remodels mitochondrial cristae in a skeletal muscle model of type 2 diabetes mellitus
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Mitochondria and Endoplasmic Reticulum (ER) contact sites (MERCs) are membrane domains that house specialized proteins that have been associated with Insulin-Resistant Type 2 Diabetes Mellitus (T2DM). T2DM subjects manifest perturbed lipid trafficking and mitochondrial DNA (mtDNA) copy number, and MERCs have been implicated in controlling these changes. Interestingly, decreased levels of Dynamin Related Protein 1 (DRP-1) may be associated with T2DM. DRP-1 plays a crucial role in mitochondrial
fission and is recruited to the mitochondrial outer membrane to promote membrane division. MtDNA nucleoids in mammalian cells are temporally and spatially linked to MERCs destined for mitochondrial division, the same membrane sites to which DRP-1 is recruited. We hypothesized that cellular depletion of DRP-1 may perturb the association of mtDNA nucleoids with ER-linked mitochondrial division sites, with feed-on effects for mitochondrial lipid homeostasis and respiratory capacity. Skeletal muscle plays an important role in the pathogenesis of TD2M, yet MERC structure and how it may relate to mtDNA distribution have not been well-defined in this cell type. To address this, we have developed a skeletal muscle model for investigating the impacts of MERC remodelling on mtDNA maintenance in post-mitotic tissues. We used Cre-lox P technology to ablate DRP-1 from murine skeletal muscle myoblasts and myotubes. Western blot analysis confirmed the deletion of DRP-1 in myotubes and demonstrated that loss of DRP-1 increased the abundance of ER stress markers, GRP-78 and FGF-21 in myotubes. A mitochondrial stress test revealed increased basal OCR, ATP-linked OCR, maximum capacity, and spare capacity in DRP1 depleted myotubes. We used fluorescence microscopy to evaluate ER, mitochondrial and lysosomal morphology in this system. Loss of DRP-1 in myoplasts increased mitochondrial volume and decreased mitochondrial number and sphericity. Lysotracker staining demonstrated that loss of DRP-1 in myoplasts increased lysosomal area and decreased lysosomal staining intensity. DRP-1 ablation led to an increase in ER surface area and lysosomal area in addition to mitochondrial length and increased MERC distance. Strikingly, visualization of organelle ultrastructure via transmission electron microscopy (TEM) confirmed the presence of extensive mitochondrial cristae remodelling in DRP-1 Cre cells. We are now poised to examine how DRP-1-dependent endomembrane remodelling, particularly cristae remodelling, impacts mtDNA replication, distribution, and turnover in skeletal muscle.

P849

**Acetylated tubulin is a functional component of the mitochondrial synapse**


Cell organelles are often engaged in homotypic and heterotypic interactions. Both mitochondria fusion and contacts between the ER and mitochondria occur preferentially on acetylated microtubules (MTs), a subset of stable MTs that together with detyrosinated MTs function as preferential tracks for conventional kinesin. In addition, two properties of acetylated MTs i.e. stability and flexibility make them uniquely adapted to sustain mechanical stress caused by organelle/organelle interactions.

Whether α-tubulin acetylation at homotypic and heterotypic mitochondria contacts is regulated by the organelle interaction machinery and/or is a functional component of the mitochondria “synapse” remains largely unknown. We found that fibroblast deprived of stable modified MTs due to loss of IQGAP1 expression, exhibited both mitochondria and ER-mitochondria communication defects that could be rescued by restoring normal levels of acetylated but not detyrosinated tubulin in these cells. We investigated whether tubulin acetylation was regulated by the mitochondria interaction machinery. A key regulator of mitochondria homotypic fusion and ER-mitochondria tethering is mitofusin-2 (MFN2), a protein mutated in the inherited neuropathy Charcot-Marie-Tooth type IIa (CMTIIa). Here we show that loss of MFN2 reduced the levels of acetylated but not detyrosinated tubulin while increasing MT and actin dynamics in immortalized MEFs. Remarkably, rescue of tubulin acetylation levels by
Pharmacological inhibition of histone deacetylase 6 (HDAC6) corrected the defect in MT dynamics, mitochondria distribution and oxygen consumption. Conversely, inhibition of HDAC6 was not able to rescue MAM activity in MFN2 KO, suggesting that control of acetylated tubulin by MFN2 may be critical for homotypic but not heterotypic mitochondria interactions. We explored the mechanism of this regulation by examining levels and distribution of the enzymes catalyzing the tubulin acetylation/deacetylation cycle and found that MFN2 interacted with ATAT1 at mitochondria, and loss of MFN2 mislocalized ATAT1 into the nucleus. These data strongly indicate that MFN2 may be part of a machinery that directly targets ATAT1 to sites of mitochondria/mitochondria contacts and that this function may be disrupted in CMTIIa. In line with interpretation, we found that loss of MFN2 reduced acetylated tubulin levels while inducing axonal degeneration in adult DRG neurons and that these defects were completely rescued by HDAC6 inhibition. Our findings reveal a novel role for MFN2 in regulating homotypic mitochondria interactions through direct targeting of tubulin acetylation and suggest that disruption of tubulin acetylation/deacetylation cycle may contribute to the onset of CMTIIa caused by mutations in MFN2.

**Focal Adhesions and Invadosomes**

P850

**srGAP1 uncouples tumor cell proliferation and dissemination in breast cancer**

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Signaling molecules that regulate axon guidance and neuronal migration have aberrant expression in many different cancers; however, their contribution to disease progression is relatively unknown. srGAP1, a Slit-Robo GTPase activating protein that regulates neuronal migration and morphogenesis, is expressed in triple negative breast cancer cells. Using dSTORM imaging, we find that srGAP1 localizes to invadopodia (invasive structures that degrade the extracellular matrix). siRNA-mediated depletion of srGAP1 results in increased extracellular matrix degradation and 3D cellular invasion in collagen I plugs. Our data demonstrates that this effect is due to the formation of more mature invadopodia with a higher turnover frequency. To analyze effects of srGAP1 on tumor cell dissemination in vivo, we developed an orthotopic mouse model of triple-negative breast cancer. Surprisingly, tumors with stable-depletion of srGAP1 have a defect in tumor growth, but are capable of metastatic dissemination. Utilizing intravital multiphoton imaging, we demonstrate that srGAP1-depleted tumor cells invade locally at a higher velocity and travel farther distances than control cells. To measure extravasation events, we used a transgenic zebrafish model with labelled vasculature Tg(kdr1:RFP) and find that srGAP1-depleted tumor cells have more extravasation events than control cells. Furthermore, in the lungs of the orthotopic mouse model, srGAP1-depleted tumor cells are more efficient in seeding than control cells, relative to primary tumor size. RNASeq data demonstrates that genes upregulated in srGAP1-depleted tumors (when compared to control tumors) are enriched in GO categories and KEGG pathways related to both the actin cytoskeleton and cell cycle. Our preliminary data suggests that srGAP1 loss results in a GO/G1 arrest, potentially through an increase in Smad2/3 signaling. In addition, Cobl, an actin-regulatory protein, is upregulated in srGAP1-depleted cells and promotes invadopodia
maturation. Taken together, our results suggest that srGAP1 is able to regulate a switch between proliferative and invasive phenotypes in tumor cells using a novel signaling axis.

P851

**Invadopodia formation requires septin 9-mediated coordination of TKS5 and MMP-14 targeting to the plasma membrane**

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Metastatic cancer invasion is facilitated by actin-rich protrusions termed invadopodia, which protrude into and degrade the extracellular matrix (ECM) by secreting matrix metalloproteinases (MMPs). Invadopodia formation involves the membrane recruitment and clustering of the scaffold protein TKS5 (Tyr kinase substrate with five SH3 domains), which along with cortactin and other actin regulators promote the invadopodia extension. However, the molecular mechanisms that underlie and regulate TKS5 and MMP targeting to invadopodia are not well understood. An increasing number of studies show that over-expression of Septin 9 (SEPT9), a member of the septin family of GTPases, enhances the migratory and invasive properties of cancer cells. However, the role of Septin 9 in invadopodia formation is poorly understood. Using a transmigration and fluorescent gelatin degradation assays, we show that SEPT9_i1 is enriched in distinct domains of nascent and mature invadopodia. On 2D gelatin matrices, SEPT9_i1 localized at areas of degraded gelatin with more enrichment in smaller areas of degradation. In 3D matrices, SEPT9_i1 localized at the basal domains of shorter invadopodia, and at distal/apical regions of longer, mature invadopodia. Using SEPT9 isoform-specific shRNAs, SEPT9_i1 depletion decreased ECM degrading activity in MDA-MB-231 cells without impacting overall cell area. Strikingly, SEPT9_i1 depletion diminished TKS5 and phosphorylated (pY421) cortactin clusters on the ventral membranes of MDA-MB-231 cells. These phenotypes were accompanied with abnormal clustering of MMP-14 at peripheral sites of the cell membrane edge, which were also devoid of the exocyst component Exo70, indicating a defect in the vesicular delivery of MMP-14 to the plasma membrane. Additionally, TKS5, MMP-14 and the Exo70 were observed to accumulate in the nucleus, which might be due to a breach of the nuclear envelope or alterations in nucleocytoplasmic shuttling. Taken together, our results show that SEPT9 is required for TKS5 and MMP-14 targeting to plasma membrane sites of invadopodia formation, and may be involved in the coordination of the nuclear and plasma membrane pools of invadopodia components.

P852

**Cell-ECM interactions regulate cell sorting and cooperation during collective invasion and metastasis**

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Cancer metastasis results in the growth of secondary tumors in vital organs and is the principal cause for cancer-related deaths. To date, most cell biology research focused on the metastasis of mesenchymal cancer cells that invade the extracellular matrix (ECM) and further disseminate via the blood stream individually. However, few recent studies in animal models and in patients suggested that cancer cells
can enter the blood circulation as clusters, highlighting the need to determine the mechanisms by which cancer cells can invade and intravasate as a group. Here, using the 3D spheroid invasion assay, we show that the invasion of cohesive clusters of 4T1 cancer cells is dependent on ECM-degrading invadopodia structures. In addition, upon mixing of the 4T1 cell line with the mesenchymal and non-invasive syngeneic cell line 67NR, we found that 4T1 cells “sorted” from 67NR cells by moving to the spheroid-ECM interface and initiated the cooperative invasion of both cell lines. Surprisingly, cell sorting in spheroids was controlled by cell-ECM interactions, namely cell-ECM adhesion strength and ECM degradation by invadopodia, and not by differential cell-cell adhesion strength, as previously reported in tissue patterning studies. Finally, we show how cooperative invasion can affect metastatic outcomes. In conclusion, for the first time, our results suggest that invadopodia enable the collective invasion of breast cancer cells. Next, we demonstrate that a cell sorting event is required for the cooperative invasion of cancer cells. Our data implicate that a few cells with invadopodia can assume leading roles and are sufficient to drive the overall invasion of cell clusters. This work emphasizes the importance of targeting invadopodia to prevent the invasion and the metastasis of collectively invading cancer cells.

P853

Mechanical cues from the microenvironment regulate Caveolin-1 Tyr14 phosphorylation distinctly in focal adhesions and mediate integrin-dependent cellular responses in murine fibroblasts

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Cells respond to distinct mechanical cues from their microenvironment that are in part mediated by mechanical and biochemical properties of the extracellular matrix (ECM). This is primarily mediated by integrins, that are vital cellular mechanosensors. Integrin-mediated cell-ECM adhesion regulates, among other processes, membrane raft trafficking through caveolae to control anchorage-dependent signalling. Caveolae are distinct plasma membrane invaginations formed by transmembrane protein Caveolin1 (Cav1) that support endocytosis and are also known to be mechanosensitive. Caveolar endocytosis is dependent on phosphorylation of Cav1 on its tyrosine-14 residue (pY14Cav1), its cellular levels thought to be unaffected by adhesion. We however now find Tyr14 phosphorylation of Cav1 is regulated by cell-ECM adhesion in mouse fibroblasts. pY14Cav1 levels significantly reduce on loss of adhesion and are restored upon re-adhesion, similar to the known regulation of focal adhesion kinase phosphorylation (pY397FAK). Caveolae isolated by detergent-free biochemical fractionation from adherent and suspended cells show comparable pY14Cav1 levels, despite a change in cellular levels. This suggests adhesion to not affect caveolar pY14Cav1, suggesting its impact to be prominently on focal adhesion associated pY14Cav1. We confirm pY14Cav1 to be prominently detected in isolated focal adhesions from re-adherent cells. Re-adhesion mediated recovery of pY14Cav1 levels is significantly inhibited upon inhibition of FAK, further confirming its regulation by integrin mediated adhesion. Integrins help fibroblasts sense increasing 2D polyacrylamide matrix stiffness and regulate pY14Cav1 levels similar to pY397FAK. PF-228 mediated inhibition of FAK, though comparable across increasing stiffness, differentially affects Cav1 phosphorylation but not that of Paxillin (pY118Paxillin). This suggests the FAK-Cav1 crosstalk to be sensitive to changing integrin-mediated signalling. Taken together, these findings highlight the presence of a FAK-mediated regulation of Cav1 phosphorylation downstream of integrins, that is vital to cellular responsiveness to adhesion-dependent biochemical and mechanical cues.
**P854**

**Synthetic dysmobility screen identified an integrated STK40-YAP-MAPK system controlling adhesion cytoskeletons**

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It remained unclear how various cell signals were integrated to control cytoskeletons during cell migration. We thus conducted a “two-hit” cell migration screen to perturb sheet migration using short hairpin RNA (shRNA) and small molecules inhibitors simultaneously in endothelial and epithelial cancer cells. Among our candidates, Serine-Threonine Kinase 40 (STK40), a putative serine/threonine kinase, showed potential interaction with MAPK in that suppressing both genes, a dramatic suppression of cell motility occurred. Here we report that STK40 affects migration via focal adhesion. STK40 knockdown results in enhanced focal adhesion (FA) intensity, FA turnover, increased stress fibers and phosphomyosin light chain (p-MLC). These phenomena can be rescued by STK40 overexpression even when its kinase domain is truncated. Our further elucidation of its molecular mechanism revealed that inhibition of MAPK signaling abolished the effect of shSTK40 on FA dynamics, symbolizing that STK40 cooperated with MAPK signaling in FA regulation. Moreover, STK40 knockdown reduced nuclear translocation of YAP, which cooperates with CREB, the downstream player of MAPK signaling, regulating cell adhesion and cell migration. Altogether, our studies demonstrate an integrated STK40-YAP-MAPK system controlling adhesion cytoskeletons during cell migration.

**P855**

**Phosphorylated paxillin and FAK constitute subregions within focal adhesions**

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Integrin-mediated adhesions are convergence points of multiple signaling pathways. Their inner structure and their diverse functions can be studied with super-resolution microscopy. We used structured illumination microscopy (SIM) to analyze spatial organization of proteins within adhesions. We found that a wide range of adhesion-localized proteins, including paxillin, are found continuously throughout adhesions. In contrast, phosphorylated paxillin (pPax) and focal adhesion kinase (FAK) form spot-like, spatially defined clusters within adhesions that are clearly separated from each other. Thus, paxillin shows distinct spatial organization within single focal adhesions based on its phosphorylation status. This difference in spatial organization could be confirmed in several cell lines. Live-cell super-resolution imaging revealed that pPax-FAK clusters persist over time but modify distance to each other.
dynamically. Additionally, we manipulated cell contractility by chemical and genetic interference by using vinculin knockout cells. These experiments revealed that spacing between single pPax clusters is mechanosensitive and actively regulated by cells. Thus, in this work we introduce a new structural organization within focal adhesions and demonstrate its regulation and dynamics. These findings might point to a self-organization mechanism on the nano-/micrometer-scale creating distinct signaling hubs within cells.

**Mechanical Forces in Development**

**P856**

*Caenorhabditis elegans* PIEZO Channel Coordinates Multiple Reproductive Tissues to Govern Ovulation

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PIEZO1 and PIEZO2 are newly identified excitatory mechanosensitive proteins. Dysfunction of PIEZOs cause a variety of genetic diseases, including the dysplasia in the cardiovascular, respiration, and connective tissues. However, the cellular and molecular mechanisms of PIEZOs in these diseases are less understood. In this study, we took advantage of the reproductive tract of *C. elegans*, which is an attractive mechanosensory model with a number of stretch-sensitive and responsive cells, to better characterize the functional roles of PIEZOs *in vivo*. A number of deletion alleles and missense mutants of PEZO-1, a sole ortholog of PIEZOs in *C. elegans*, caused severe defects in reproduction, including a reduced brood size, sperm number and ovulation rate. *In vivo* imaging observations show that oocytes undergo a variety of transit defects as they enter and exit the spermatheca during ovulation. Post ovulation oocytes were frequently damaged during spermathecal contraction. Given that PIEZO is a non-selective ion channel and may regulate spermathecal contractility through Ca2+ signaling pathways, we tested the genetic interactions between *pezo-1* mutants and several cytosolic Ca2+ regulators with RNA interference (RNAi). Indeed, *pezo-1* interacts genetically with known regulators of calcium signaling. Depletion of Ca2+ channels *itr-1* and *orai-1*, and Ca2+ transporting ATPase *sca-1*, by RNAi substantially enhanced the reproductive deficiencies in *pezo-1* mutants. Additionally, the *pezo-1* mutants displayed a sperm navigational defect. Sperm that were readily washed out of the spermatheca during ovulation failed to migrate back to the spermatheca. In hermaphrodite, sperm is guided to the spermatheca by the F-series prostaglandins, which synthesis requests a precursor from intestinal yolk granules. The observation of a defective yolk granule endocytosis in the *pezo-1* mutants suggests that the prostaglandin synthesis may also be disrupted, which led to the defective sperm attraction. Using an auxin-inducible degradation system, we depleted PEZO-1 in different tissues. Reduced brood sizes were observed in each tissue-specific degradation strain, suggesting PEZO-1 may act in different reproductive tissues to coordinate the reproduction. Lastly, the genetic suppressor and chemical antagonists screen will be performed in patient-specific alleles to suppress the reproductive defects as a readout.
Mechanical activation of mitochondrial respiration and biogenesis during stem cell differentiation

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Proliferative stem cells favor glycolysis that reserves carbon sources for the generation of building blocks, while differentiated cells perform mitochondrial respiration to power cellular activities. A metabolic transition from glycolysis to respiration, together with an increase in mitochondrial/mtDNA content, occur during stem cell differentiation. However, the underlying mechanism is still poorly understood. Our previous studies using *Drosophila* female germline stem cell as a model uncovered that a transient JNK activation in the differentiating cyst induces the transcription of nuclear-encoded mitochondrial genes related to respiration, and mtDNA replication and expression. Differentiating germline cysts are compressed by the surrounding somatic follicle cells (FCs), suggesting an increase in mechanical tension. Here, we find that, in differentiating cells, mechanosensation by the Tmc ion channel activates JNK and mitochondrial respiration through acute increase in cytosolic Ca\(^{2+}\). Our further genetic analysis demonstrates that an osmotic stress responsive signaling is required for the activation of JNK by high cytosolic Ca\(^{2+}\) in differentiating cells. Importantly, female flies with impaired germline mechanosensation produce eggs with less mitochondrial content and display lowered fertility. In vivo, stem cells differentiation is accompanied by altered mechanical properties of environment. Therefore, we propose that the mechanoresponsive mechanism in differentiating cells plays a general role in the metabolic transition from glycolysis to mitochondrial respiration, which is vital for tissue development.

Engineering anteroposterior axis in 3D human neuroepithelium tissue guided by mesendoderm

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Human pluripotent stem cells (hPSCs) have been demonstrated to be capable of differentiation towards neural lineages with self-organizing capability. Three-dimensional multicellular human neural organoids models have been developed to better recapitulate spatial-organization of the developing neural tissues. However, organoids generated from current protocols suffer inconsistency in size and morphology. In development in vivo, tissues are ordered according to the major axes in the embryo. One of the major axes along which the developing neural tissue are patterned to includes the anteroposterior axis. Formation of the anteroposterior axis in the neural tissues results from dynamic interplay between germ layers. The objective of this study is to demonstrate anteroposterior patterning of organized neuroepithelium (NE) tissues facilitated by mesendoderm (ME) tissues surrounding them. In our results, we show that geometrically confined hPSCs in the form of micro-patterns could differentiate to form spatially organized NE tissue surrounded by ME tissues using a neural differentiation protocol preceded by pulsed primitive streak induction. After 2 days of differentiation, a PS-like population (BRY\(^+\)) can be seen segregated from NE progenitors (SOX2\(^+\)) in a folding structure. A ME population of different anteroposterior identities is derived from the primitive streak-like population induced. Further culture of the micro-patterned tissues for 6 days gives a continuous NE layer (PAX6\(^+\)) surrounded by the ME population in the core of the tissue. The micro-patterned tissues can be matured up to 14 days to generate NE organoids with distinct anteroposterior pattern marked by HOX markers.
When the initial PS induction given to the micro-patterned hPSCs was altered to produce mainly anterior ME (FOXA2⁺), NE organoids formed subsequently do not display spatial organization. Our results shed lights on how ME facilitates organization of NE tissues with the establishment of an anteroposterior axis.

P859

Control of osteoblast regeneration by a train of Erk activity waves
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Regeneration is a complex chain of events that restores a tissue to its original size and shape. How are cells coordinated across the large sizes of regenerating adult tissues for proper morphogenesis? Signalling gradients established by diffusable morphogens regulate cell behaviour in myriad multicellular systems and contexts and have been proposed to direct regenerative events. In addition, feedback mechanisms in biochemical pathways can provide an effective mechanism of communication across large distances, but how they might regulate growth during tissue regeneration remains largely unresolved owing to difficulties in imaging, analyzing and conceptualizing these complex systems. To overcome those barriers, we developed the regenerating scale of adult zebrafish as a quantitative platform to study tissue growth and morphogenesis. Scales are external millimeter-sized bony disks coated with a mono-layer of bone-depositing osteoblasts. After scale loss, the osteoblast tissue regenerates in just a few weeks. Scales are an ideal system to study tissue regeneration quantitatively owing to their simple organization and accessibility to live imaging. We discovered that traveling waves of Erk activity instruct hypertrophic growth of the regenerating osteoblast tissue. Using a combination of theoretical and experimental analyses, we find that Erk activity propagates as concentric trigger waves across the millimeter-sized scale in few days and induce patterned tissue growth, thus orchestrating scale morphogenesis. Furthermore, periodic induction of synchronous, tissue-wide, Erk activation in place of travelling waves impairs tissue growth, suggesting that wave-distributed Erk activation is key to regeneration. Our findings reveal trigger waves as a regulatory strategy to coordinate cell behavior and control tissue morphogenesis during regeneration.

P860

Distinct Functions of Krebs cycle metabolites in modulating mitochondrial dynamics, stem cell survival and pluripotency
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The conversion of the metabolic demands between glycolysis and oxidative phosphorylation remodels embryonic stem cell (ESC) pluripotency and differentiation. Metabolites can impact cell fate through epigenetic landscapes such as TCA cycle metabolite α-ketoglutarate promotes histone and DNA demethylation in maintaining pluripotency. However, whether the other Krebs cycle metabolites can affect pluripotent status have not been comprehensively characterized yet. Among the eight Krebs cycle metabolites, we identified citrate, succinyl CoA, oxaloacetate and malate led to pluripotent human ESC death. Isocitrate strikingly promoted ESC differentiation and rescued the cellular death caused by oxaloacetate in ESC and in mouse early embryos. In vitro manipulation of the exit of ESC pluripotency, mitochondria changed from punctate into the filamentous network. The differentiated hES and
isocitrate treatment counteracted the oxaloacetate-caused cell death by restoring cristae structure and elongating mitochondrial configuration. These results suggest that mitochondrial membrane structures and dynamics remodeled during the exit of pluripotency and Krebs cycle metabolites have distinct functions in modulating stem cell pluripotency and survival.

P861

**Actinomyosin Remodeling is Necessary for a Naïve Human Embryonic Stem Cell State**

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Despite significant work on the transcriptional and epigenetic programs that regulate human embryonic stem cell (hESC) pluripotency, the cell biology of dynamic changes in pluripotency states remains poorly understood. Contrasting with mouse embryonic stem cells which are maintained in a ground state of self-renewing naïve pluripotency, clonal hESCs are typically maintained in a state of self-renewing primed pluripotency resembling the post-implantation blastocyst. However, recently defined culture conditions allow the conversion of primed hESCs to a naïve ground state, which opens new opportunities for insights into the initial differentiation processes and regulators of hESC biology. We found that during the conversion of hESCs from primed to naïve pluripotency, there is a striking and previously unreported actinomyosin filament remodeling where a “fence” of bundled contractile actin filaments encloses naïve colonies. This actinomyosin fence is decorated with phosphorylated myosin light chain and the ERM protein moesin but not ezrin and is tethered to actively remodeled adherens junctions, as indicated by changes in E-cadherin and β-catenin localization. These observations suggest that the actinomyosin fence exerts a tensional force that is transmitted through adherens junctions to neighboring cells. Consistent with a predicted tensional unit, the Hippo signaling effector TAZ is predominantly nuclear in naïve hESC colonies but predominantly cytosolic in primed hESCs. We also found that activity of Arp2/3 complex but not formins is necessary for formation of the actinomyosin fence. Pharmacologically inhibiting Arp2/3 complex activity with CK666 blocked assembly of the actinomyosin fence, led to retention of TAZ in the cytosol, and prevented relocation of moesin from diffuse in the cytosol to enriched along cortical actin filaments. CK666 also blocked key naïve pluripotency characteristics, including increased expression of the naïve cell markers PECAM1 and ESRRB, determined by qPCR, and impaired self-renewal as determined by colony formation. These data were consistent across two different hESC lines and two different conversion media. Taken together, our findings identify a previously undescribed actinomyosin architecture in naïve hESCs with suggested mechanosensitive signaling as indicated by nuclear translocation of TAZ, which promotes the acquisition of functional nativity during transit between these cell states. These results provide new insights into understanding the cell biology of pluripotency in human stem cells, with further implications related to understanding early human development and the use of hESCs in therapeutic strategies. Funded by NIH GM116384, CA197855 and T32 5T32HD007470-23.
Uncovering novel roles for AHR and tryptophan-derived molecules in intestinal repopulation
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The intestine maintains rapid replenishment of its lining by orchestrating fast cell proliferation and differentiation. Misregulation of these processes can lead to cancer and inflammatory bowel diseases (IBD), including colitis. Metabolites derived from dietary components and the gut microbiome contribute to the maintenance of the intestinal lining. Some metabolites found in the intestinal environment are kynurenine (Kyn), FICZ, and indole which function as ligands for the transcription factor aryl hydrocarbon receptor (AHR). AHR is best known for its role on regulating the expression of xenobiotic enzymes. Recent experiments in our lab revealed that AHR and its tryptophan-derived metabolite, Kyn, play an important role in cell growth, and thus AHR may act as a central integrator of growth signaling. However, the specific molecular mechanism by which AHR and these small molecules affect the proliferation of stem and progenitor cells and therefore intestinal regeneration is poorly understood. My goal is to define the role of AHR and its ligands in intestinal repopulation using primary intestinal organoids and decellularized colons as model systems. I hypothesize that AHR ligands will facilitate the repopulation of afflicted intestine by activating AHR, and that AHR is physiologically important for regeneration of the intestinal lining. In a preliminary experiment, I found that silencing AHR abolished the ability of colonic transformed cells to repopulate a decellularized colon scaffold. Nevertheless, proliferation of these cells is not affected in culture. Because loss of AHR prevented colonic repopulation with colonic cancer cells, I hypothesize that depletion of AHR will also prevent repopulation of colonic scaffolds by normal primary colonic cells and organoids. Additionally, I predict that activation of AHR with its endogenous ligands, will facilitate repopulation of colonic scaffolds by normal primary colonic cells. Understanding the importance of nutrient-derived small molecules in colonic repopulation will shed light on how the intestine responds to different environmental signals and may lead to new therapies to treat IBD. This work will lay the foundation for future work using organoids derived from patients with IBD. The knowledge obtained from this project, can help us design tools and protocols to regulate the activation of AHR by modulating its ligands to induce the ability of the cells to grow and regenerate damaged intestinal tissue.

Mechanical bistability during Drosophila mesoderm invagination
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Actomyosin-mediated apical constriction is a common mechanism that drives localized folding in epithelia. The way by which contractile stress generated near the apical surface of an epithelial sheet drives out-of-plane folding of the sheet is not fully understood. We investigated this question using Drosophila mesoderm invagination as a model. During Drosophila gastrulation, the mesodermal cells constrict apically and subsequently invaginate from the surface of the embryo. To test whether actomyosin contractility is required throughout the folding process, we developed an optogenetic tool to acutely inhibit non-muscle myosin II ("myosin") by controlling the membrane recruitment of a dominant negative form of Rho1 (Rho1DN). Blue light-mediated plasma membrane recruitment of Rho1DN resulted in rapid Rho1 inhibition, as evidenced by the immediate loss of apical myosin in the
apically constricting cells. With this tool, we examined the effect of myosin inhibition at different stages of mesoderm invagination. We found that inhibiting myosin before the transition from apical constriction to invagination ("T\textsubscript{trans}") resulted in immediate relaxation of the constricted cells. In contrast, inhibiting myosin after T\textsubscript{trans} did not delay the progression of invagination. The bipartite response to myosin inhibition suggests that \textit{Drosophila} mesoderm invagination is a mechanically bistable process. Computational modeling demonstrated that mechanical bistability during invagination can arise from a buckling-like mechanism that depends on compressive stress from the flanking lateral ectodermal tissues. In support of this model, laser cutting in live embryos showed that the lateral ectodermal tissues are indeed compressive before T\textsubscript{trans}. Furthermore, disrupting the lateral ectoderm using laser ablation or mechanically decoupling mesoderm from ectoderm led to a specific arrest of mesoderm invagination at T\textsubscript{trans}. Combining mutant analysis with computational modeling, we demonstrated that a buckling-like mechanism can increase the robustness of folding process and enable mesoderm invagination when apical constriction is impaired. Taken together, our data suggest that compressive stress from the lateral ectodermal tissues facilitates mesoderm invagination by triggering mechanical bistability during the folding process.

**Mechanisms of Myogenesis**

P863

**Microtubules enable cardiac growth via localization of mRNA and translational machinery**

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The terminally differentiated muscle cells of the heart undergo constant contraction throughout the lifetime of an organism. These cells must maintain homeostasis of the contractile unit (sarcomere) while also retaining the ability to rapidly remodel in response to a changing environment. This is especially important in times of growth, either in functional or disease states, when the cardiomyocyte (CM) requires the addition of new sarcomeres. However, it is still unclear how precisely new sarcomeres are formed, added, and remodeled within the existing structure. And though the transcriptional landscape of the heart has been studied extensively during hypertrophy, there is a dearth of information regarding the spatiotemporal regulation of these transcripts. We aim to uncover the mechanisms of mRNA transport in the CM, and how these mechanisms regulate growth of the cell. Our data suggests that microtubules are required for proper trafficking of mRNA in the CM. Consistent with previous reports, we find that transcripts are enriched at the sarcomere and intercalated disc of the CM using smFISH. Treatment with the microtubule-destabilizer colchicine, both \textit{in vitro} and \textit{in vivo}, causes a perinuclear collapse of mRNA. Proper localization is also disrupted by kinesin-1 knockdown. Additional pathophysiologically relevant manipulations of microtubules, such as overexpression of microtubule-associated proteins (MAPs) or modulation of microtubule post-translational modifications (PTMs), phenocopies the effect of colchicine. Disruption of the actinomyosin network does not change mRNA localization. We find that a growth stimulus (phenylephrine, PE) induces a peripheral localization of sarcomeric transcripts in growing cells, but both this specific localization and growth of the cell is blocked by colchicine treatment. We speculated that this sub-localization of transcripts might mirror
organization of translational machinery. Accordingly, we demonstrate that ribosomes and nascent proteins also localize to the sarcomere both in vitro and in vivo, and this localization is similarly disrupted with colchicine treatment. Concomitant with mislocalized translation, protein degradation is increased in the presence of colchicine. We hypothesize that the CM uses a strategy of local translation whereby transcripts are actively transported to distal locations in the cell and locally translated by ribosomes for insertion into new sarcomeres. Microtubule disruption causes mislocalized translation and increased degradation. In the future, we seek to understand where precisely the addition of new sarcomeres occurs in the cell, and to determine the fate of both transcripts and proteins that are not properly trafficked, translated and incorporated during growth of the cell.

P864

**Calcium signaling as a global regulator of myoblast differentiation and fusion**

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Myoblast fusion is crucial for the regeneration of adult vertebrate skeletal muscle fibers. Impaired myoblast fusion is correlated with various muscle dystrophies, causing muscle fiber degeneration. Recent studies have identified TMEM8C (a.k.a MYMK) and Gm7235 (a.k.a MYMX) as the first muscle-specific membrane proteins essential for myoblast fusion and together sufficient to induce fusion between cells that normally do not fuse. We have used MYMK and MYMX to identify additional proteins involved in regeneration and myoblast fusion using a two-pronged approach combining in silico promoter analysis with transcriptome analysis. We initially searched for genes with homologous promoters to mammalian MYMK and MYMX promoters. In parallel, we performed RNA sequencing to identify differentially expressed genes between differentiated and undifferentiated primary mouse myoblasts. This approach enabled the identification of genes which were both previously implicated in myoblast fusion as well as novel genes. These putative genes include not only the muscle-specific proteins but also genes that exhibit a ubiquitous expression pattern. The gene ontology (GO) analysis of a section of the genes shared between the two lists suggested that these are involved in Calcium signaling and excitation-contraction coupling. To test whether the identified genes indeed affect myoblast differentiation and fusion, we knocked down the expression of the calcium-signaling associated genes in myoblasts prior to induction of differentiation. Our preliminary results shown that disrupting the regulation of cytosolic calcium leads to hyper fusion without affecting differentiation. These results point to the active involvement of calcium signaling upstream of MYMK and MYMG in the regulation of myoblast fusion in primary mouse myoblasts.

P865

**Embryonic myosin replacement in myofibrils is controlled through degradation and ubiquitination by a ubiquitin ligase, Ozz.**

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Ubiquitin proteasome system (UPS), one of the major protein degradation systems, plays an essential role in turnover of myofibrillar proteins in skeletal muscle. Myofibril proteins are highly organized into the minimal contraction unit sarcomere, which is comprised of Z-bands, thin filaments, and thick
filaments. Approximately 300 myosin polymerize to form a single thick filament. In our previous study, we showed that approximately 50% of myosin in thick filament is replaced within 3 h by fluorescence recovery after photobleaching technique. Although the UPS is required for myosin degradation in skeletal muscle cells, how the UPS affects myosin replacement process remains unclear. Here, to address this, we investigated the effect of ubiquitin ligase on myosin replacement in muscle cells isolated from pectoral muscle of chick embryo. Ozz is a muscle specific ubiquitin ligase which recognizes embryonic myosin heavy chain (Myh3) as a substrate. Overexpression of Ozz significantly decreased replacement rate of eGFP tagged Myh3 (eGFP-Myh3) and increased ubiquitinated myosin to be degraded in myotubes, suggested the shortage of replaceable myosin supply due to enhanced degradation of ubiquitinated Myh3. In contrast Myh3, replacement rates of other myosins (Myh1 and Myh7) were not changed in myotubes overexpressing Ozz, indicating the specificity of Ozz for Myh3. Intriguingly, a USP inhibitor MG132 treatment decreased replacement rate of eGFP-Myh3, though MG132 enhanced accumulation of ubiquitinated myosin in the cytosol, suggesting that ubiquitinated myosin is unlikely incorporated into the myofibrils. In conclusion, Ozz selectively controls Myh3 replacement rate through ubiquitination and degradation of Myh3.

P866

Cell states beyond transcriptomics: integrating structural organization and gene expression in hiPSC-derived cardiomyocytes

The Allen Institute for Cell Science is developing a state space of stem cell structural signatures to study changes in cellular organization as human induced pluripotent stem cells (hiPSCs) differentiate into cardiomyocytes. In this study we combined quantitative image-based readouts of gene expression, transcript localization, and cellular organization to evaluate the changes in hiPSC-derived cardiomyocytes during the early/intermediate stages of differentiation. One of the goals of this study was to understand the relationship between gene expression and structural organization by integrating both measurements in single cells. To investigate changes in gene expression during these stages, we first performed single-cell RNA sequencing on cardiomyocyte populations collected at D12, D24 and D90. We used a cluster independent bootstrapped sparse regression statistical approach to rank differentially expressed genes based on their ability to correctly assign cells to the transcriptionally similar D12 or D24 populations. From this ranked list we identified a subset of genes to use as probes for RNA fluorescent in situ hybridization (FISH) to measure transcript abundance. These included known developmentally regulated myosin heavy chain isoforms MYH6 and MYH7. We also developed novel, scalable machine learning methods for classifying and quantifying sarcomeric organization in single cells, which improves upon manual annotation. To determine whether the changes in gene expression found in single-cell RNA sequencing correspond to differences in sarcomere organization, we combined high resolution imaging using the mEGFP-ACTN2 cell line with multiplexed RNA-FISH. This co-analysis of quantitative single-cell measurements of gene expression and sarcomeric organization revealed that while the expression of some genes such as MYH7 were positively correlated with increased sarcomeric organization, both measurements are required to describe cell state. The image-based nature of this analysis also revealed subcellular transcript distribution patterns, such as sarcomeric localization of MYH7 transcripts. While we did not find a relationship between transcript localization and myofibril
organization in these cells, further analysis of transcript localization with structure may provide insights into this relationship. These findings suggest that cells may be better characterized by a combination of spatial and quantitative measurements of transcripts and structures rather than by either one alone. While we demonstrated this concept in cardiomyocytes during early differentiation, integrating gene expression and structural organization can be applied more broadly to create a more complete understanding of cell behavior and states.

P867

**Structured-surface cultureware promotes myotube alignment and improves in vitro assay robustness**

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**Objective:** We assessed the utility of nanopatterned plates and high-content analysis for studying a dystrophic phenotype and defining myotube growth patterns *in vitro*. **Approach:** Healthy and dystrophic human skeletal myoblasts were differentiated on nanopatterned cultureware to promote linearly aligned myotube formation. High-content analysis was used to quantify expression of markers specific to mature myotubes. Caspase 3/7 activity was tracked using live-imaging to assess apoptosis. **Results:** In contrast to the healthy control, dystrophic myotubes displayed maturation defects evidenced by decreased expression of critical structural and functional proteins, and a sharp spike in apoptosis 24 hours post-serum withdrawal which steadily increased throughout the differentiation process. **Conclusions:** Myoblasts cultured and differentiated on structured-surface cultureware results in physiologically relevant myotube morphology. This platform enabled us to develop improved assays for investigating relevant readouts at scale and robustness needed for drug discovery.

**Microtubule-Associated Protein Structure Studies**

P868

**Atomic model of MAP7-microtubule interaction**

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Microtubule-associated proteins (MAPs) can regulate all aspects of microtubule (MT) function, from MT dynamics, to interaction with motor proteins involved in organelle transport, cell polarity or axon morphogenesis. All MAPs possess a MT-binding domain that anchors them to the MT surface, and often a disordered region that extends from the MT and that has recently been proposed to drive formation of liquid condensates for some MAPs. A large variety of MT-binding domains have now been identified and their footprints on the MT surface are an area of active research in an effort to understand their effect on MTs and their interplay with one another. Structural MAPs and MT-based motors can compete for real state on the MT or can have a more synergistic effect on each other. For example, most structural
MAPs appear to be inhibitory to kinesin-1, with the exception of MAP7, which contains a unique kinesin-1 interaction domain. To learn more about the MAP7 binding to MTs and how it relates to that of other MAPs and to motors, we have used cryo-EM to visualize MAP7 on MTs and to generate an atomic model of its interaction with tubulin. Like tau, MAP7 binds along protofilaments and its footprint on the MT extends over both intra and interdimer interfaces. But MAP7 binding site, which involves a conserved, long alpha helical segment on MAP7, does not overlap with that of tau, and only partially overlaps with that of kinesin via the N-terminal portion of the long alpha helix of MAP7. However, the cryo-EM density of that MAP7 region of the helix is poor, indicating potential flexibility that might be used by MAP7 to accommodate the co-binding of kinesin-1.

Cryo-EM snapshots of MT dynamics and regulation
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Microtubules are an integral part of eukaryotic cells, performing cellular functions necessary for structural integrity, intracellular transport, motility, and cell division. One hallmark of microtubules (MTs) is their dynamic instability, a rapid interplay of growth & shrinkage at the plus-end. Our research utilizes Cryo-EM to understand the mechanisms behind the regulation of MT dynamics. One method of regulation lies within the inherent properties of tubulin, such as GTP hydrolysis and how one hydrolysis event is communicated throughout the entire MT lattice. We hypothesize that a GTP-cap of the microtubule is structurally distinct from the GDP lattice based on the fact that end- binding proteins specifically bind near the end of the MT. We use a catalytically dead mutants and GTP-analogs to better understand the GTP-state and cooperativity of GTP-hydrolysis. Extrinsic factors such as MT-associated proteins (MAPs) can also regulate MT dynamics. For example, Kif21B can completely pause MT growth and catastrophe. To better understand the molecular mechanism behind this pause-factor, we determined the structure of a domain of Kif21B bound to the microtubule.

Microtubules assembled in vitro are composed of discontinuous mixed lattices
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Microtubules are commonly described as hollow tubes made of 13 protofilaments in which αβ-tubulin heterodimer are aligned in a head-to-tail-like fashion, and as 3-start left-handed helices that follow the lateral interactions between tubulin monomers. These lateral interactions are thought to occur between homotypic monomers (α-α, β-β; denoted the “B-lattice”), except at a particular lattice region called the seam, which involves heterotypic interactions (α-β, β-α; denoted “A-lattice”). In this study, we have asked whether microtubules assembled in vitro can contain several seams. Toward this aim, we have decorated microtubules with kinesin heads and analyzed their structure using dual-axis cryo-electron tomography followed by sub-tomogram averaging. In agreement with previous studies, we observed that such microtubules are built from a variety of protofilament and helical-start numbers configurations. Strikingly, we find that while the majority of the lateral tubulin interactions are indeed of
the B-lattice type, microtubules can contain multiple-seams of the A-lattice type. Detailed analysis of individual microtubules revealed that the location and the number of seams can vary along the microtubule wall, giving rise to mixed AB-mosaic lattices. Changes in seam configuration along individual microtubules imply the presence of holes of at minima the length of a tubulin monomer inside their lattices.

P871

Cryo EM sheds light on how katanin severs microtubules
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The AAA ATPase katanin uses the energy of ATP hydrolysis to sever microtubules, cytoskeletal polymers with a flexural rigidity comparable to Plexiglas. Katanin is present in all metazoans and its function is important in many fundamental biological processes including cell division, centriole biogenesis, neuronal morphogenesis and phototropism. Its mutation causes microcephaly in humans. The microtubule templates katanin hexamerization and activates its ATPase. The structural basis for these activities and how they lead to severing is unknown. Here, we show that the β-tubulin tail is necessary and sufficient for microtubule severing. Cryo-EM structures reveal the essential tubulin tail glutamates gripped by a double spiral of electropositive loops that line the katanin central pore. Each spiral couples allosterically to the ATPase and binds alternating, successive substrate residues in the tubulin tail, with consecutive residues coordinated by adjacent protomers. This tightly couples tail binding, hexamerization and ATPase activation. Structures of the katanin hexamer in different conformations suggest an ATPase-driven, ratchet-like translocation of the tubulin tail through the central pore. Moreover, we find an essential disordered region outside the AAA core that anchors katanin to the microtubule. Thus, katanin uses multivalent interactions with the microtubule through its disordered linkers and the ordered AAA motor core to exert the forces necessary to extract tubulin dimers out of the microtubule and sever it.

P872

Cm1-driven assembly and activation of yeast γ-tubulin small complex underlies microtubule nucleation
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Microtubule (MT) nucleation is regulated by the γ-tubulin ring complex (γTuRC), conserved from yeast to humans. In Saccharomyces cerevisiae, γTuRC is composed of seven identical γ-tubulin small complex (γTuSC) sub-assemblies which associate helically at the spindle pole body to template microtubule growth. Spc110p, a distant pericentrin homologue, recruits γTuSC to the nuclear face of the spindle pole body, while Spc72p recruits it to the cytoplasmic face. γTuRC assembly provides a key point of regulation for the MT cytoskeleton. Previously published data suggest a hierarchical model of γTuSC activation; with γTuSC assembling at the SPB into an open γTuRC that would be further activated by closure. Here we combine cross-linking mass spectrometry (XL-MS), X-ray crystallography, and cryo-EM structures of monomeric and dimeric γTuSC and open and closed helical γTuRC assemblies in complex with Spc110p.
to elucidate the mechanisms of γTuRC assembly. γTuRC assembly is substantially aided by the evolutionarily conserved CM1 motif in Spc110p spanning a pair of adjacent γTuSCs. By providing the highest resolution and most complete views of any γTuSC assembly, our structures allow phosphorylation sites to be mapped, suggesting their role in regulating spindle pole body attachment and ring assembly. We further identify a structurally analogous CM1 binding site in the human γTuRC structure, which allows for the interpretation of significant structural changes arising from CM1 helix binding in metazoan γTuRC structures.

**Modifications in Cell-Cell and Extracellular Signaling**

**P873**  

**Analyzing the mechanism of the inhibitory effect of Lfng on Jag1-Notch1 signaling**  

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Notch signaling is a highly conserved juxtacrine signaling across species that controls cell fate decisions in many tissues. Notch receptor is highly glycosylated by multiple enzymes. Among them, Fringe, an N-acetylglicosamine transferase is known to regulate the ligand selectivity of Notch receptor. Lunatic fringe (Lfng) promotes Delta-like (DII) ligand-induced signaling through enhancing DII-Notch interaction while inhibits Jagged (Jag)-induced signaling, but the binding affinity between Jag1-Notch1 is enhanced by Lfng. In this regard, the detailed mechanisms of how Lfng changes the selectivity remain to be clarified. Since cleavage of Notch1 intracellular domain (NICD) is critical for activation of Notch signaling, we firstly examined the cleaved NICD level by western blotting and found that the NICD level was reduced by Lfng modification of Notch1. Next, we focused on transendocytosis, endocytosis of Jag1-Notch1 complex into Jag1 expressing cell, which is thought to generate the pulling force and subsequent cleavage of NICD. We performed an endocytosis assay using the soluble form of Notch1 (Notch1-Fc) which is artificially clustered by antibody. The internalization levels of Notch1-Fc which was glycosylated by Lfng (Lfng/Notch1-Fc) into Jag1 expression cells were comparable with those of Notch1-Fc which is not glycosylated by Lfng. We next examined the ubiquitination of Jag1, which is required for endocytosis of Jag1, and the ubiquitination level of Jag1 induced by Notch1-Fc was not altered by Lfng modification of Notch1. These results suggest that Lfng modification does not affect the Jag1 endocytosis induced by Notch1-Fc. Thus, we hypothesized that Lfng changes a state of Notch1 clustering. To examine a clustering state of Notch1, we performed sucrose gradient ultracentrifugation of lysates from Notch1 expressing cells co-cultured with or without Jag1 expressing cells. We found that the distribution of Notch1 extracellular domain tended to be changed to lower fraction of the gradient by Lfng modification only when co-cultured with Jag1 expressing cells. These results suggest that excessive Notch1 clustering induced by Lfng might play an important role for inhibiting Jag1-Notch1 signaling.

**P874**  

**A lipid-dependent molecular relay conducts Sonic hedgehog ligand between sending and receiving cells to effect Hedgehog signaling**  

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Hedgehog signaling governs critical processes in embryogenesis, adult stem cell maintenance, and tumorigenesis. The activating Sonic hedgehog (SHH) ligand is posttranslationally modified by palmitate and cholesterol, two lipids that tether SHH strongly to the membrane of producing cells. In many contexts, however, SHH signals to faraway target cells, raising the question of how SHH is mobilized to act at a distance. Here, we investigate the biochemical basis for SHH movement between producing (“sending”) and responding (“receiving”) cells. We demonstrate that the secreted SCUBE2 protein acts as a SHH chaperone, binding the lipid moieties on SHH to permit ligand release from sending cells. The soluble SCUBE2-SHH complex, although highly potent in cellular assays, cannot directly signal through the SHH receptor, Patched (PTCH1). Rather, signaling by SCUBE2-SHH requires a molecular relay mediated by the coreceptors CDON/BOC and GAS1, which cooperate to relieve SHH inhibition by SCUBE2. CDON/BOC bind both SCUBE2 and SHH, recruiting the complex to the receiving cell surface. SHH is then handed off, in a dual lipid-dependent manner, to GAS1, and, finally, from GAS1 to PTCH1, initiating signaling. Together, our results reveal that SCUBE2, CDON/BOC, and GAS1 comprise a novel extracellular chaperone system that channels SHH from the sending cell membrane to the PTCH1 receptor via successive handoffs of the SHH lipid appendages. This distributed SHH reception mechanism, which permits sensitive and tunable signal detection, defines a new key step in Hedgehog signal transduction and, more generally, a paradigm for coreceptor function upstream of a primary receptor.

P875

Saccharomyces cerevisiae share exosomes for altruistic protection from heat stress

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Exosomes are critical mediators of intercellular communication. Composed of complex macromolecules, they are a subtype of extracellular vesicle released from donor cells into interstitial fluids. Here, they can travel long distances before being selectively recognized and internalized by recipient cells to elicit diverse physiological responses, from immune cell activation to tissue development. Exosomes also contribute to disease, from coordinating cancer cell metastasis to spreading prion proteins underlying neurodegeneration. But despite their importance to human health, the molecular basis of exosome biology remains enigmatic. However, exosome communication is not limited to humans. It mediates cellular processes in all eukaryotes, including Saccharomyces cerevisiae (baker’s yeast). Although there are few reports on yeast exosome biology, the biogenesis machinery is conserved, as are many payload proteins, including heat shock protein chaperones. Given the genetic tractability of S. cerevisiae, we reasoned that it would be an exceptional model to better understand exosome biology. Here, we tested the hypothesis if exosomes are involved in the yeast heat shock response. This required designing a new conditioning paradigm, whereby high-density yeast cultures were subjected to pulses of sublethal heat stress followed by subsequent lethal heat stress. We found that a single pulse was sufficient to confer protection from lethal heat stress, by measuring cell viability using methylene blue. Next, we fused GFP to Bro1, an exosomal protein, to track exosome release. Upon sublethal heat stress, we found that intracellular GFP-positive puncta, representing exosome stores, disappeared using fluorescence microscopy. This coincided with the detection of Bro1-GFP in exosome fractions isolated by ultracentrifugation. Further characterization revealed preparations containing intact vesicles with an
average diameter of 115 nm, confirming exosome enriched samples. Finally, we collected Bro1-GFP positive exosomes released upon sublethal heat stress and added them to naïve yeast cells. We show their internalization and protective ability upon lethal heat stress. Thus, we conclude that exosomes contribute to the heat shock response in yeast, revealing an altruistic mechanism for population survival, and offering new insight into how eukaryotic cells maintain proteostasis.

P876

Cell Surface Labeling by Engineered Extracellular Vesicles
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Extracellular vesicles (EVs) can mediate local and long-range intercellular communication via cell surface signaling. In order to perform in vivo studies of unmanipulated, endogenously released EVs, sensitive but stringent approaches able to detect EV-cell surface interactions are needed. However, isolation and reinfusion of EVs can introduce biases. A rigorous way to study EVs in vivo is by genetically engineering membrane-bound reporters into parental cells. Still, the amount of reporter molecules that EVs can carry is relatively small, and thus, the sensitivity of the approach is suboptimal. In this work, we address this issue by engineering EVs to display a membrane-bound form of Sortase A (SrtA), a bacterial transpeptidase that can catalyze the transfer of reporter molecules on the much bigger surface of EV-binding cells. To this end, we designed 4 different SrtA expression vectors and selected the most efficient one. We then optimized reaction requirements, including calcium dependence, substrate chemistry and target protein sequence. For example, cell surface labeling by EVs was enhanced by the presence of 5 N-terminal glycines on the plasmamembrane. To test whether substrate affinity was driving EV binding to target cells, we performed 2 independent tests based on the results of the reaction requirements above. We did not observe aspecific targeting and achieved efficient in vitro labeling of EV-binding cells, even toward native proteins (i.e. carry only one N-terminal glycine). As compared to indirect labeling of EV-binding cells (e.g., using CD63-GFP reporter), the SrtA-based approach shows 1-2 log increase in sensitivity. This novel strategy will be useful to identify and study the full set of host cells interacting with native EVs in vivo. For example, tumor-derived EVs are known to accumulate in lymph nodes, but a clear understanding of which immune cells are influenced by tumor EVs is not yet available, nor are the signaling outcomes.

P877

The Major Sperm Protein (MSP) domain of the VAPB/VPR-1 protein is cleaved and secreted in C. elegans
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VAPB is a type-II ER transmembrane protein whose N-terminal Major Sperm Protein (MSP) domain is cleaved and secreted. In humans, secretion of the MSP domain has been linked to Amyotrophic Lateral Sclerosis (ALS). The P56S substitution mutation within the VAPB protein segregates with cases of familial ALS and prevents the secretion of the MSP domain. Since the MSP domain faces the cytosol, rather than the ER lumen, how it is cleaved and secreted is not yet known. This work used C. elegans to study how the VAPB N-terminal MSP domain is proteolytically processed and secreted. VPR-1 is the C. elegans
homolog of mammalian VAPB. *C. elegans* null for *vpr-1* are sterile and can be rescued by *vpr-1* expression in the neurons, germline, or intestine. Overexpression of VPR-1 with an N-terminal FLAG tag revealed that the N-terminal VPR-1 peptide is secreted from intestinal cells to bind the distal gonad. Immunofluorescent imaging of endogenously tagged VPR-1 revealed a polarized localization pattern of VPR-1 termini in intestinal cells. In addition, western blots of these endogenously incorporated epitope tags revealed two stable VPR-1 products. Mass spectrometry determined that the smaller of the two products is the cleaved N-terminal peptide of VPR-1 that spans the MSP domain and ends at a conserved leucine at the 156th amino acid position. An RNAi screen of 422 genes using *C. elegans* was conducted to identify genes required for MSP domain cleavage or secretion. This screen identified a v-SNARE and several proteasome components as potential mediators of VPR-1 MSPd proteolysis and trafficking. Further analysis is needed in order to verify these candidates. In all, results from this work advanced our understanding of the MSP domain cleavage and secretion and may pave the way to understanding ALS pathogenesis.

**Molecular Pathways in Gametogenesis**

**P878**

**A meiotic switch in lysosome acidity supports spermatocyte development in young flies but collapses with age**

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Animal fertility requires the production of functional gametes. Understanding fundamental mechanisms that direct animal gametogenesis, and how they go awry with age, may reveal new entry points to combat infertility. Recently, we found that lysosomes activate during oocyte meiotic maturation in *C. elegans*, suggesting that a developmental switch in lysosome activity promotes female germ-cell health in young animals. Whether lysosomes are similarly regulated during sperm development is unknown. Using *Drosophila melanogaster*, we report that lysosomes activate as spermatocytes enter meiosis and prohibit spermatocyte multinucleation. As in other contexts, this abnormal developmental phenotype appears linked to membrane destabilization; our data indicate that spermatocyte lysosomes turn over E-cadherin at endocytic foci, minimizing membrane-associated E-cadherin accumulation at ectopic sites. Importantly, we found that diminished lysosome acidity immediately precedes E-cadherin build-up and germ-cell multinucleation. Thus, loss of lysosome activity may contribute to age-related infertility in males. These findings demonstrate that lysosome activity is tightly linked to meiotic progression in both male and female germ cells and hint that lysosomes may be key determinants of male reproductive aging.

**P879**

**Small heat shock protein 27 acts as a critical determinant in the maintenance of male germline stem cells homeostasis under cadmium stress: A Drosophila based study**

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In the recent years, studies have shown that stem cells can experience different kind of stress from environmental chemicals perturbing their normal functions. The physiological relationship has been well documented stating the role of heat shock proteins (HSPs) in normal stem cells behavior. However, studies involving chemical stress in alteration of HSPs expression which may lead to the impairment in stem cell homeostasis are poorly understood. Environmental toxicant, Cadmium (Cd), perturbs crucial biological process such as spermatogenesis resulting in male infertility/subfertility. Thus, for combating Cd stress, maintenance of the adult male germline stem cells (GSCs) homeostasis becomes essential in order to maintain spermatogenesis and hence male fertility and HSPs may play an important role for the same. Therefore, the present study was aimed to evaluate the role of HSPs in male GSCs homeostasis under Cd stress using Drosophila testis as an in vivo model. Under developmental exposure of Cd (20 μg/ml), a significant elevation in reactive oxygen species (ROS) level was seen at the apical region of the testis of exposed (20 μg/ml Cd) organism as compared to control. The study also showed a significant reduction in the number of GSC in the testis of exposed group as compared to control. The resulting testes showed precocious and incomplete differentiation of GSCs. In the apical region of the testis of exposed Drosophila, significant alteration in small HSP 27 expression levels was observed among various other screened HSPs as compared to control group. Genetic screening study altering HSP 27 expression revealed that the adhesion of GSCs at the stem‐hub interface was affected under Cd stress which ultimately results into the loss of GSCs and causes precocious differentiation. In summary, the study infers the elevated levels of ROS resulted in altered HSP 27 expression reducing GSCs number which was accompanied by inappropriate differentiation leading to disturbed GSCs homeostasis in Drosophila males. This finding provides a basis of chemical (Cd) stress induced dysregulation in HSP 27 expression and also suggests that small HSP 27 plays a critical role in the maintenance of male GSCs homeostasis.

The study further advocates Drosophila as an alternative animal model for evaluating the role of HSPs in stem cells homeostasis under chemical stress.

P880

**Cell and Nuclear Growth Dynamics in Developing Drosophila Germline Cysts**


Across species, from insects to mammals, the future fertilizable egg develops within a cluster of sister germ cells that are interconnected through cytoplasmic bridges. To meet the biosynthetic demands of the developing oocyte and future embryo, germ cells within these clusters grow dramatically, and do so in a differential, yet coordinated manner. As opposed to the known growth scaling laws that govern single cells, the dynamics that direct the coordinated growth of these developing cell clusters remains poorly understood. Here, we use the Drosophila egg chamber, a germline cluster of sixteen cells connected in a highly reproducible fashion, to explore the collective growth dynamics of individual cells and organelles over multiple orders of magnitude of size increase. Through a supervised learning algorithm for the automatic reconstruction of cell clusters from 3D stacks of images, we have created a quantitative high-throughput pipeline for systematically isolating and measuring relevant properties of individual cells within each cluster. This work establishes the framework for applying machine learning techniques for the analysis of differential growth rates in other multicellular systems and highlights new approaches for analyzing large datasets of information to address longstanding questions in biology.
Actomyosin cytoskeletal control of syncytial germline architecture in *C. elegans*

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Animal embryogenesis depends on disproportionately large oocytes that contain the biomass necessary for cleavage divisions and morphogenesis before the animal accesses extracellular nutrients. Many germlines promote the formation of large oocytes by allowing the exchange of cytoplasm among cell-like compartments of a syncytium. The oogenic syncytial germline of *C. elegans* is an elongated C-shaped tube in which peripheral germ cell compartments surround a central core of common cytoplasm, the rachis. Germ cells open to the rachis via intercellular bridges (rachis bridges) in their apical surface. Cytoplasm flows from germ cells near one end of the germline towards and into a subset of germ cells that enlarge and eventually become oocytes. Both rachis bridges and germ cells’ apical domains that comprise the rachis lining are enriched for components of the cortical actomyosin cytoskeleton. It has been suggested that oogenesis is regulated by both rachis bridge size and overall contractility of the rachis lining. To gain a better understanding about how these two factors coordinate to promote oogenesis, we measured the abundance of nine conserved structural and regulatory elements of the actomyosin cytoskeleton. The density of many of these elements on both the rachis bridges and the rachis lining was non-uniform throughout the germline. To test whether these protein abundance dynamics represented significant increases and decreases, and how abundance dynamics correlate with germ cell development, we used SiZer (SIgnificant ZERo crossing of the derivatives) statistical analysis to identify germline regions with statistically significant changes in rachis bridge perimeter. Subdividing the germline into distinct regions based on the changes in rachis bridge size allowed us to identify various significant correlations between change in protein distribution and rachis bridge size. Proteins implicated in contractility did not increase in density in germline regions of diminishing rachis bridge size, suggesting that rachis bridge dynamics, e.g. closure, are not simply regulated by actomyosin contractility on their perimeter. These observations suggest that regulation of germline architecture and oogenesis rely on a balance of distribution of contractile components on rachis bridges and rachis lining, as well as other factors including cytoplasmic flows.

On the junctional protein Afadin: from spindle positioning to intestinal organoids development in a Wnt-dependent manner.

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Colorectal cancer (CRC) is one of the deadliest human malignancies where the high rate of relapse is increased by the high frequency of cancer stem-like cells (CSC). Aberrancies in stem cell proliferation in the intestinal epithelium are key in the generation of CSCs, as well as alterations in pathways controlling mitotic spindle-alignment and cell polarity. The Wnt signaling pathway, which is constitutively active in several CRCs, is a critical regulator of intestinal stemness. Interestingly, recent studies identified a role for Wnt signals in oriented stem cell division. However, the molecular mechanisms governing ISC divisions and how they are deregulated in CRC remains largely unclear. In our study we report that the ablation of the junctional and actin-binding protein Afadin by lentiviral RNA infection leads to defective
morphogenesis of intestinal organoids derived from wild-type mice. These defects are characterized by a significant decrease in the differentiation rate and a reduced proliferation compared to control organoids. Consistently, Afadin-depleted organoids are characterized by differences in the expression of specific cell fate markers with an increase in stem cells factors (such as Olfm4) compared to differentiative ones (such as Alp1). In addition, preliminary data on whole transcriptome revealed a deregulation of pathways implicated in the regulation of mitotic processes, cell cycle and small GTPases signaling, suggesting that Afadin plays also a role in transcriptional activation. Lastly, we observed impairment in the mitotic spindle orientation of dividing cells in the intestinal crypts, which is in agreement with the role of Afadin in spindle positioning. Our data suggest that Afadin plays a role in the molecular machinery orchestrating Wnt-dependent planar divisions and transcriptional programs in the murine intestinal epithelium, this way contributing to organogenesis. Importantly, our results provide molecular information on niche-regulated intestinal stem cell divisions that maintain epithelial morphogenesis under physiological conditions and are likely deregulated in neoplastic conditions.

Oncogenes and Tumor Suppressors: Cell Cycle and Genomic Instability

P884

A cytosolic RNA binding protein in Chlamydomonas functions as a cell-cycle repressor in the retinoblastoma cell-size control pathway

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Coordination of growth and division in eukaryotic cells is thought to be mediated by cell-size checkpoints, but the mechanisms for size homeostasis outside of fungal models are largely unknown. The green alga *Chlamydomonas reinhardtii* divides by a multiple fission cell cycle, where a long period of cell growth is followed by a rapid series of cell divisions to produce \(2^n\) daughter cells. Two control points are used to couple cell size to cell cycle progression: the Commitment checkpoint ensures that enough growth has taken place to allow completion of at least one division cycle, and the DNA synthesis/mitosis (S/M) checkpoint couples mother cell size to cell division number to ensure that daughter populations are uniform regardless of starting mother cell-size. The *tny1−1* mutant was identified in a forward insertional mutagenesis screen and exhibits a recessive small-size phenotype due to defects at both the Commitment and the S/M size checkpoints. *TNY1* encodes a predicted hnRNP A-related RNA binding protein with two N-terminal RNA recognition motifs (RRMs) and a low complexity glycine-rich C-terminus. Immunofluorescence showed that TNY1 is cytosolic throughout the cell cycle. Quantitative immunoblotting to detect TNY1 protein from synchronous cell populations revealed that daughter cells are born with a fixed amount of TNY1 whose absolute abundance remains constant on a per-cell basis during G1 phase, but whose overall cellular concentration decreases as cells grow. *TNY1* mRNA and protein levels peak during cell division and are reset to their highest concentration in newly-formed daughters. Altering the dosage of *TNY1* in diploids or by overexpression impacted daughter cell size, indicating a quantitative relationship between *TNY1* protein levels and mitotic size control. Epistasis experiments placed *TNY1* upstream of the cyclin dependent kinase CDKG1 [1], whose substrate is the MAT3/RB (retinoblastoma tumor suppressor homolog). CDKG1 is produced just before cells divide and is eliminated upon mitotic exit, but in *tny1−1* strains post-mitotic CDKG1 remains detectable, suggesting that TNY1 inhibits CDKG1 production or turnover. *In vitro* RNA binding assays showed that recombinant
TNY1 can bind to the unusually long and uridine-rich 3' UTR of CDKG1 mRNA but not to the CDS region or 5' UTR. Taken together, our data suggest a model where TNY1 influences cell-size homeostasis at least in part through dosage-dependent repression of the activator CDKG1, possibly mediated through direct binding of TNY1 to the CDKG1 3'UTR. Experiments to test this model are ongoing. [1] Li, Y.*, Liu, D.*, Lopez-Paz, C., Olson, B.J., and Umen, J.G. (2016). A new class of cyclin dependent kinase in Chlamydomonas is required for coupling cell size to cell division. eLife 5, e10767

P885

**STING-Mediated Cell Death in Synergetic Killing of Ovarian Cancer Cells Co-treated with Paclitaxel and SYK Inhibitor**

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Paclitaxel, a microtubule-targeting drug, is one of the most commonly used chemotherapy to treat ovarian cancer. However, the majority of ovarian cancer patients eventually develop paclitaxel resistance, resulting in the dismal outcome for these patients. We have discovered that the hyperactivity of Spleen Tyrosine Kinase (SYK) in ovarian cancer is correlated to paclitaxel resistance. It was observed that inhibiting SYK activity leads to paclitaxel killing of cancer cells otherwise resistant. We also demonstrated that SYK inhibition enhances the mitotic arrest, by decreasing phosphorylation of microtubule-associated proteins in paclitaxel-treated dividing cells, resulting in higher cell death. However, it is not clear how SYK inhibition makes non-dividing paclitaxel-resistant cancer cell susceptible again. To unveil the mechanism in question, we co-treated SKOV3 cells, an invasive human ovarian cancer cell line, with paclitaxel and R406, a small molecule inhibitor of SYK. By fluorescence microscopy, we observed pronounced fragmented morphology of the nucleus in the co-treated cells. In particular, 66% cells co-treated with SYK inhibitor and paclitaxel exhibit the fragmented nucleus morphology, while only approximately 15% cells in the paclitaxel treatment only, R406 treatment only and vehicle control groups exhibit fragmented nucleus. Interestingly, we found that the fragmented nucleus is also prevalent in cofilin-silenced cells. Cofilin is an SYK substrate and a severing protein of actin filaments. Moreover, DNA leakage is detected in 85% cells co-treated with SYK inhibitor and paclitaxel, while it is only detected in approximately 36% cells in the paclitaxel treatment only, R406 treatment only and vehicle control groups. We examined stimulator of interferon genes (STING) signaling in the treated groups, known to be activated by cytoplasmic DNA, such as the leaked DNA from the fragmented nucleus, and to trigger downstream cell death. As expected, STING signal is detected in the cells co-treated with SYK inhibitor and paclitaxel but not in the control groups. In summary, we demonstrate that SYK inhibition may enhance the killing effect of paclitaxel in ovarian cancer cells via a novel mechanism involving interaction between cofilin and microtubules, which causes damages of the nucleus, leading to genomic DNA leakage and then STING activation. This new mechanism may provide more molecular targets in addition to SYK as co-treatment to boost paclitaxel cytotoxicity in chemoresistant ovarian cancer.
Frequent inactivation of the mitotic duration sensor in p53-functional cancers
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When mitosis is prolonged beyond a threshold time, the mitotic duration sensor—which requires the ubiquitin protease USP28 and the p53-binding protein 53BP1—is activated, leading to p53 stabilization and growth arrest. Inhibiting PLK4, the kinase that controls centriole duplication, allows to assess the functionality of the mitotic duration sensor, because centrosome loss prolongs mitosis by slowing assembly of the mitotic spindle. Prior work showed that non-transformed cells with an intact p53 pathway and mitotic duration sensor upregulate p53 and arrest when centrosomes are removed, whereas cancer lines that lack an intact p53 pathway continue to divide, albeit at a slower rate due to mitotic errors. These observations prompted us to address if cancers with an intact p53 pathway (~50% of all cancers) retain the mitotic duration sensor and whether sensor status dictates their response to mitotic inhibitors. To address these questions, we assessed the effect of centrosome removal and functionality of the mitotic duration sensor in 14 p53-positive cancer cell lines from 9 different cancer types. All 14 cell lines abruptly ceased proliferation when p53 was stabilized by inhibition of its antagonistic ubiquitin ligase MDM2. By contrast, response to centrosome removal varied significantly and correlated with functionality of the mitotic duration sensor. 5 tested cell lines had an intact mitotic duration sensor and abruptly ceased proliferation upon centrosome loss. 4 cell lines had no detectable mitotic duration sensor and continued to proliferate following centrosome loss, while the remaining 5 cell lines had a leaky mitotic duration sensor and exhibited intermediate growth. Thus, the mitotic duration sensor is suppressed in a significant fraction of p53-positive cancers and the status of the sensor is a predictor of the response of p53-positive cancers to anti-mitotic drugs. Molecular analysis indicates that sensor inactivation occurs by multiple mechanisms including mutation of USP28 and 53BP1 and genetic hyper-activation of the phosphatase WIP1, a negative regulator of p53 signaling. Finally, we show that cancer lines with an inactive mitotic duration sensor are prone to chromosome missegregation, despite a functional p53 pathway. Based on these results, we suggest that an oncogenic event common to p53-positive cancers is inactivation of the mitotic duration sensor, which may facilitate tolerance of genomic instability. Our results also indicate that mitotic duration sensor status predicts cancer-specific sensitivity to anti-mitotic drugs, with possible implications for personalized therapeutic approaches.
depolymerization to induce apoptosis. APC is also involved in the regulation of microtubule stability through direct binding with tubulin and the plus end binding protein EB1. These roles suggest that APC gene expression may have significant implications on the efficacy of PTX in breast cancer treatment. We previously created APC knockdown cells (APC shRNA1) using the human TNBC cell line, MDA-MB-157. Using these cells, we have determined that APC loss-of-function significantly increases resistance to 1PTX. We predicted that varying microtubule stability in normal cells versus APC shRNA cells may lead to a difference in cell cycle protein modulators during G2/M transition. We first performed cell cycle analysis using flow cytometry, which showed increased PTX-treated APC shRNA1 cells in the G2/M phase compared to the PTX-treated parent cells. These data suggested that APC status does not prevent G2/M arrest after PTX treatment. The G2/M transition proteins cyclin B1 and CDK1, including the inhibitory (Thr\(^{14}\) and Tyr\(^{15}\)) and activating (Thr\(^{161}\)) phosphorylation sites on CDK1, were analyzed by western blot. Analysis showed that the APC shRNA1 cells have increased total CDK1, with no change in cyclin B1 or the phosphorylation patterns of CDK1. Given that the complex of cyclin B1 and CDK1 is only active when the two proteins are in the nucleus, we examined the subcellular localization of these cell cycle protein modulators. We have identified that CDK1 is preferentially localized to the cytoplasm while cyclin B1 has no clear cellular localization preference. Other cell cycle proteins involved in PTX sensitivity were examined by western blot. Analysis showed significant upregulation of CDK6 and a modest increase in p27 expression in APC shRNA1 cells. PTX treatment increased cyclin D1 protein levels in both parent and APC shRNA1 cells. RNA-sequencing was performed on APC shRNA1 cells to show overall changes in gene expression following APC knockdown. Up- and down-regulation of cell cycle-related genes was seen in the APC shRNA1 cells when compared to the parental. Examination of these cell cycle modulators can further our understanding of PTX resistance in APC shRNA1 cells and help to identify potential therapeutic targets. Future studies will examine combination therapies or genetic approaches to overcome PTX resistance.

P888

Analysis for expression pattern of cell senescence associated genes on DLD-1 cells treated with Anisomycin

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Anisomycin, which is originally discovered as an antibiotics drug, has potent effect of activation of c-Jun N-terminal kinase (JNK). It is reported that JNK signaling cascade induces excessive endoplasmic reticulum (ER) stress resulting in apoptosis, anisomycin has been expected as chemotherapy for hepatocellular carcinoma, osteosarcoma, or leukemia. We previously showed that activation of the JNK signaling cascade causes proteolysis of GATA-6, which is promising treatment target of colorectal cancer, on DLD-1 cell lines. In this study, DLD-1 cells were treated with anisomycin then the proliferation of them was measured. The RNAs extracted from anisomycin-treated DLD-1 cells were also analyzed for gene expression pattern with RNA-sequence (RNA-seq) method. We demonstrated that anisomycin remarkably inhibits the proliferation of DLD-1 cells via G2/M arrest in a plate culture. It did not induce apoptosis under growth arrest conditions. The growth of DLD-1 cells in a spheroid culture was suppressed by anisomycin. Furthermore, results of analysis for differentially expressed genes (DEG) for each time series, we observed that JNK cascade genes (c-Jun, MKK4) were transiently over-expressed at 3 h after anisomycin-treatment and so were the cell senescence associated genes such as CDKN2. On the other hand, CDKN2, HSP90 and HSP70 showed high expression level at 24 h after treatment and the
cytoskeletal, adhesion associated genes (Laminin, Desmocollin, Netrin) were significantly downregulated. These results suggest that the suppressive effect of anisomycin on DLD-1 proliferation might be triggered by activation of cell senescence associated genes and the decrease of cytoskeletal compartment lead growth arrest.

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**Chromosome Copy Number Alterations affect Cell Phenotype**


Nearly all human tumors undergo Copy Number Alterations (CNA), which may result in the loss or gain of entire chromosomes during DNA replication and division. This chromosomal variation can be expected to change gene expression and may impact cellular phenotypes while also enhancing disease progression. Partial or whole deletion of chromosome 5 has been frequently identified as a CNA in multiple cancers. Notably, chromosome 5 contains multiple tumor suppressor genes, including Adenomatous polyposis coli (APC), Nucleophosmin 1 (NPM1), and Lysine-specific demethylase 3B (KDM3B). APC has previously been identified as a highly significant tumor suppressor whose loss is common in colorectal and lung cancers. To study the effect of chromosome 5 loss, we compare an A549 human lung adenocarcinoma clone with three copies of chromosome 5 (P3) to a genetically identical A549 clone which has lost a copy of chromosome 5 (N3). We hypothesized that loss of a single copy of chromosome 5 will exhibit a gene expression dosage effect, confer a proliferative advantage to N3 cells, and produce other observable phenotypic differences. Chromosome 5 deletion was first identified through a live cell reporter system and confirmed with SNP array analysis and karyotyping. Single cell RNA-Seq confirms a gene dosage effect, including lower levels of APC. Using proliferation assays, including one that couples to 3D migration, we find that N3 cells proliferate more than P3 cells. Decreased APC regulation of β-catenin may play a role in altering cell phenotype. Experiments indicate that drug inhibition of the APC pathway in P3 cells results in greater proliferation, nuclear area, and nuclear roundness which mimic the effect of chromosome 5 loss seen in N3 cells. Chromosomal divergence within the original, genetically identical A549 cells with three copies of chromosome 5 resulted in clonal populations (P1-P4) with inherent differences in motility, morphology, and gene expression. Multiple pathways, including those regulated by tumor suppressors such as APC, could contribute to enhanced proliferation, altered cellular phenotype, and drive tumor progression after chromosome 5 loss.

P890

**Nap1l1 promotes telomere elongation by telomerase**

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Telomere elongation by telomerase counteracts telomere attrition during each cell cycle to sustain telomere length homeostasis required for continuous proliferation of human stem cells and >85% of cancer cells. This process is restricted to the late S/G2 phase of the cell cycle, and is controlled by regulating the recruitment of telomerase to telomeres. Cell cycle-dependent regulation of telomerase recruitment to telomere is not only critical for maintaining the telomere length equilibrium, but also for
its coupling with DNA replication. However, the molecular mechanisms underlying the cell cycle-dependent telomerase recruitment to telomeres are not fully understood. Here, we have identified nucleosome assembly protein 1-like1 (NAP1L1) as a novel hTert-associated protein in human cells. NAP1L1 is a member of histone chaperone family and its cytoplasmic-nuclear shuttling is tightly regulated during the cell cycle to facilitate DNA replication, chromatin assembly and cell proliferation. Our data indicate that NAP1L1 interacts with hTert and TRF1 through distinct functional domains, which may promote the recruitment of telomerase to telomere for telomere elongation. NAP1L1 is highly expressed in T-cell acute lymphoblastic leukemia (T-ALL) cell lines, and recurrent NAP1L1-MLLT10 fusions have been reported in T-ALL. Our data indicate that overexpression of NAP1L1-MLLT10 fusion protein promotes telomere maintenance in cancer cells, which requires both the nuclear localization signal (NLS) and OM-LZ domain from MLLT10 fusion partner. These results provide new insights into the mechanisms of telomere maintenance as well as potential strategies for therapeutic intervention of T-ALL.

P891

Oncogenic Ras alters cell shape and mechanics to facilitate cell division under confinement
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To divide within a tissue, cells requires physical space in which to assemble a mitotic spindle and segregate chromosomes. Cells generate this space by rounding and stiffening in mitosis, driven by actin filament re-arrangement at mitotic entry. We investigated the effect of oncogene activation on this process in normal epithelial cells. We found that short-term induction of oncogenic RasV12 activates downstream MEK/ERK signaling to alter cell mechanics and enhance mitotic rounding. These Ras-dependent changes allow cells to round up and divide faithfully when confined underneath a stiff hydrogel, a condition which introduces catastrophic errors in chromosome segregation in non-transformed cells. Thus, by promoting cell rounding and stiffening in mitosis, oncogenic Ras enables cells to proliferate under conditions of mechanical confinement, like those experienced by cells in crowded tumors. We are now exploring how these findings play out in tissues: how mitotic mechanics is regulated in an epithelium and how this is disrupted by oncogene activation to promote tissue structure breakdown.

P892

Knockdown of SCAMP3 Suppresses the Proliferation of Inflammatory Breast Cancer Cells through ERK/p21 Signaling
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Inflammatory Breast Cancer (IBC) is one of the most lethal forms of breast cancer (BC), where patients have a 43% increased risk of death compared to non-IBC advanced BC. We previously identified increased expression of the secretory carrier membrane protein 3 (SCAMP3) in EGFR overexpressing SUM149 IBC cells, tissues, and in the IBC, metastasis promoting structure, the tumor emboli. SCAMP3 is an endosome-associated protein that has been related to poor prognosis of glioma and hepatocellular
carcinoma patients. Recent studies have shown that knockdown of SCAMP3 decreases cell proliferation, motility, and growth of cancer cells. Thus, we aim to elucidate the role of SCAMP3 in the regulation of IBC proliferation. To investigate how SCAMP3 contributes to the IBC cellular response, we knocked down (SC3KD) the expression of SCAMP3 in IBC SUM149 and non-IBC MDA-MB-468 cells using the CRISPR/Cas9 technique. Wild type (WT) and SC3KD cells were used to assess cell proliferation at 24, 48, and 72 hours, population doubling time (PDT), and the expression of cancer proliferation-associated proteins. Our results demonstrate inhibition of SUM149 SC3KD cell proliferation, whereas SC3KD MDA-MB-468 cells showed a reduction of approximately two hours in the time of replication in comparison with WT. Immunoblotting showed that knockdown of SCAMP3 increased the expression of p21 and decreased the phosphorylation of ERK and the expression of LC3B on IBC cells. We did not observe change on MDA-MB-468 SC3KD cells. Taken together, our data show that SCAMP3 plays a promoting role in the proliferation of IBC through pro-survival signaling modulation.

**Post-Transcription Gene Regulation**

**P893**

**Uniting Cleavage: Insights into human tRNA splicing reveal separate functions for the TSEN complex and CLP1.**

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The tRNA splicing endonuclease (TSEN) complex is essential for removing introns out of intron containing tRNA in archaea and eukaryotes. In humans, the complex is comprised of two endonucleases (TSEN2 and TSEN34) and two structural proteins (TSEN54 and TSEN15) as well as an accessory protein, the 5'-hydroxyl polynucleotide kinase CLP1. Mutations in these five proteins are the leading cause of a severe genetic neurodevelopmental and neurodegenerative disease, pontocerebellar hypoplasia (PCH). Using recombinant protein and in vitro tRNA cleavage assays, we show that the human TSEN complex carries out its conserved intron cleavage activity in the absence of CLP1. Furthermore, using RNAi in *Drosophila* S2 cells, we show that RNAi of CLP1’s *Drosophila* homolog, cbc, leads to a increase in the production of tRNA intron circles (tricRNAs) and mature tRNA. This data suggests that CLP1 is a negative regulator of tRNA splicing. Supporting this finding, we further show that overexpression of kinase-active, but not kinase-deficient, inhibits tricRNA biogenesis in HEK cells. Together, our findings support a new model of human tRNA splicing in which CLP1 is associated with the TSEN complex in order to serve as a critical regulator of tricRNA and mature tRNA production. The work presented here is from our recent publication: Reconstitution of the human tRNA splicing endonuclease complex: insight into the regulation of pre-tRNA cleavage.

**P894**

**Nucleocytoplasmic shuttling of Gle1 plays a novel role at transcription termination sites by affecting localization of DDX1 and CstF-64**

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Gle1 is a master regulator of a set of RNA-dependent DEAD box ATPase proteins involved in the remodeling of messenger (m)RNA-protein complexes during multiple steps in gene expression. Roles for Gle1 are well defined at the cytoplasmic face of the NPC during mRNA export, and in the cytoplasm during translation and the stress granule response. In this study, we identified a novel nuclear role for Gle1 during transcription termination. In HeLa cells, a two-fold nuclear accumulation of poly(A)+ RNA is observed when nucleocytoplasmic shuttling of Gle1 is perturbed by uptake of a unique 39 amino acid peptide comprising of the Gle1 shuttling domain (SD). To determine the composition of the poly(A)+ RNA pool sequestered in the nucleus, HeLa cells were treated with Gle1-SD or scrambled peptide, and nuclear RNA was isolated for RNA-seq analysis of the poly(A)+ RNA library. Differential analyses comparing Gle1-SD peptide versus scrambled peptide treatments revealed a specific subset poly(A)+ RNAs enriched in the nucleus. Using click-IT chemistry for labeling nascent transcripts in-cell, treatment with Gle1-SD peptide showed increased levels of the nuclear-enriched poly(A)+ RNA subset through heightened transcription, and not through a block in nuclear export. Furthermore, we found elongated 3'-UTRs was a common feature of the enriched poly(A)+ RNAs, indicative of a transcription termination defect. To identify a potential DEAD-box protein targeted by Gle1 in the nucleus, the localization of Dbps with known transcriptional roles was analyzed in response to Gle1-SD treatment. Whereas no change was observed in DDX19B localization, Gle1-SD treatment resulted in a redistribution of DDX1 in the nucleus. Proximity ligation assays further revealed that DDX1 colocalizes with Gle1, and Gle1-SD treatment disrupts interaction between Gle1 and DDX1 and between DDX1 and the pre-mRNA cleavage stimulation factor CstF-64. An increase in nuclear R-loop signal intensity was also observed with diminished Gle1 nucleocytoplasmic shuttling, as well as colocalization of Gle1 at R-loops. Taken together, these studies suggested a nuclear role for Gle1 in coordinating DDX1 function during transcription termination. We propose that when Gle1 shuttling is disrupted, formation of the DDX1-CstF-64 complex is perturbed and pre-mRNA cleavage is not executed at the proper TTS and this results in elongation of the 3'-UTR and increased R-loop formation.

P895

Mapping Genomic Loci Driving Dynamic Protein Abundance Variation in Haploid Saccharomyces cerevisiae
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Organisms respond to environmental change by regulating RNA and protein expression of specific genes over time. This dynamic response can vary within a population as a result of natural DNA sequence variation between individuals. Historically, studies have focused predominantly how genetic variation impacts RNA level regulation while relatively few studies have probed the relationship between DNA variants and protein synthesis and decay rates. However, a growing body of evidence suggests that RNA levels are often poor proxies for protein levels in many systems, motivating research into the genetic mechanisms that control cellular protein levels directly. Furthermore, no study to date has mapped the genetic variation associated with protein levels in a dynamic system. Using mating-pheromone response in haploid yeast as a model system we employ time-based Bulk Segregant Analysis (BSA) coupled with Fluorescence Activated single-Cell Sorting (FACS) and Next-Gen Sequencing (NGS) to map protein-level Quantitative Trait Loci (pQTL), or regions of the genome associated with protein expression variation. A pilot study on the mating response gene Fig1 identified 21 distinct genomic loci associated with FIG1
protein abundance at an FDR of 5%, including some time-dependent QTL acting at specific stages of pheromone response. Current work to expand this analysis to a broader set of mating pheromone-responsive genes will allow for the identification of pQTLs that are distinct or shared across genes and physiological states. This research examines the complex nature of genotype-phenotype relationships in natural populations and improves our understanding of how molecular networks are influenced by genetic and environmental interactions over time. The observation that some pQTLs show time-dependent characteristics emphasises the importance of studying gene expression variation across multiple levels of regulation and in dynamic conditions.

P896

Codon bias and mRNA folding structure stability interact to modify protein synthesis rates

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Codon bias and mRNA folding structure stability (mF) are properties of mRNA that are known to be able to influence protein synthesis rates. Codon bias is understood to affect ribosome elongation rates based on concentrations of available cognate tRNAs for each codon in an mRNA. Although alterations in mF targeted to specific locations in mRNA reveal mixed outcomes on protein production, the overall mF of an mRNA surprisingly has a positive association with protein production via an unknown mechanism. Both codon bias and mF have the potential to be important factors influencing inter-individual differences in protein synthesis, yet little is known about their effects in a natural population. To address this open question, we investigated genome-wide how allelic differences in codon bias and mF amongst 22 isolates from the Saccharomyces cerevisiae population impact protein synthesis rates. We found that allelic variation in codon bias has a highly significant positive association with protein synthesis rates and that the association is largely driven by polymorphisms in domain encoding and C-terminal regions of the mRNA. We found that allelic variation in mF has a highly significant positive association with protein synthesis rates and that the association could not be broken down into additive localized effects within the mRNA. Lastly, we found that codon bias and mF have a synergistic interaction that is positively associated with protein synthesis rates and that when the interaction is accounted for, mF independently is negatively associated with protein synthesis rates. Our results confirm the importance of codon bias and mF in inter-individual variation in protein expression and extend our current understanding of how they interact with each other. Further investigation is required to understand the molecular mechanisms underlying their interaction.

P897

How Autophagy and Herpesvirus Collaborate for Neurodegeneration and Contribute to Alzheimer’s Disease

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Since the 1960s viral pathogenesis researchers have considered herpesviruses as an underlying factor for Alzheimer’s disease (AD). We reported molecular interactions between herpes simplex virus type 1
(HSV-1) and the amyloid precursor protein, the parent of amyloid plaques pathognomonic for AD (Sapute-Krishnan et al 2003; Chen et al. 2011). Furthermore, several studies report biochemical interactions between HSV-1 and autophagy. Using several brain banks for specimens of four brain regions in post-mortems of individuals with and without cognitive impairment prior to death. Readhead et al. 2018 found molecular-genetic evidence linking activity of 6 different human herpesviruses to AD, including HSV-1, HSV-2, HHN6, HHN7, VZV and CMV. Of these, HHN6, a common virus causing a minor childhood illness and thought to be a nuisance, came out as most significant. Using a QTL approach, a network of candidate AD-associated genes were found that correlated with viral load and activity. This network did not include consideration of autophagy genes. Our hypothesis is that viral replication and egress highjacks cellular membrane systems and thereby alters autophagic function. Those individuals carrying genetic variance that protects against this dynamic will be less vulnerable to cognitive impairment despite viral load, or viral load will be diminished. Here we apply software developed by Readhead et al., available through Synapsee.com, and have successfully downloaded the public data reported in the Readhead paper. In addition, we prepared a list of 180 autophagy-associated genes. We then determined a list of quantitative trait loci (QTL) of viral load and activity by comparing viral DNA amounts and viral gene transcription levels with host genetic variance, and secondarily correlated those to diagnosis, with or without cognitive impairment. We defined without AD as those individuals without cognitive impairment prior to death. Next we aim to mine this comparison for QTL in autophagy-associated genes and determine a network of autophagy gene variance in AD versus none-AD.
Ultimately we will mine RNASeq for these host gene products, and examine a second database of specimens from a different repository for expression levels of host autophagy genes relative to viral load. This study will identify a novel mechanism explaining relationships between herpesvirus and AD and identify new therapeutic targets. Supported by NCRR and NCATS UL1TR001449.

Prokaryotic Cell Biology

P898

High-temperature live-cell imaging of cytokinesis, cell motility and cell-cell adhesion in the thermoacidophilic crenarchaeon Sulfolobus acidocaldarius


Growing evidence suggest that eukaryotes emerged from within archaea, making these microorganisms new models in the study of evolutionary cell biology. However, significant technical challenges have limited the study of extremophile cell biology. For example, the absence of methods for performing high-resolution, live-cell imaging at high temperatures (>50°C) has impeded the study of cell motility and cell division in thermophilic archaea such as model organisms from the genus Sulfolobus. Here we describe a system for imaging samples at 75°C using high numerical aperture, oil-immersion lenses. With this system we observed and quantified the dynamics of cell division in the model thermoacidophilic crenarchaeon Sulfolobus acidocaldarius. In addition, we observed previously undescribed dynamic cell shape changes, cell motility, and cell-cell interactions, shedding significant new light on the high-temperature lifestyle of this organism.
Quantitative super-resolution imaging of efflux pumps in biofilm-associated bacteria
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The AcrAB-TolC protein complex is a multidrug efflux pump found in Enterobacteriaceae such as Escherichia coli, Salmonella species and Klebsiella pneumoniae. It is the main representative of the Resistance-Nodulation-cell Division (RND) superfamily of bacterial efflux pumps, which is of high clinical interest since most multiple drug resistance (MDR) phenotypes occurring in Gram-negative bacteria are associated with the overexpression of these proteins. A high degree of resistance to antimicrobials is also a defining characteristic of bacterial biofilms, and the possible role of efflux pump expression in this phenomenon is still unclear. Since biofilms are highly complex environments in which isogenic bacteria display widely different gene expression patterns, quantitative imaging with subcellular spatial resolution is necessary to investigate the expression dynamics of efflux pumps in this kind of bacterial communities. Here, we present a combination of photo-activated localization microscopy (PALM) and precision genome editing that allows us to directly image the spatial distribution of individual proteins in planktonic and biofilm-associated Escherichia coli, and to quantify its temporal evolution with single-molecule precision. By employing a CRISPR/Cas9-assisted recombineering approach, we can fluorescently label endogenous proteins while keeping the transcriptional regulatory mechanism intact. We apply our technique to the study of the AcrB component of the AcrAB-TolC efflux pump, and monitor its expression over time during biofilm development. We find that the amount of AcrB instances present in cells significantly drops when bacteria switch from a planktonic to a biofilm-associated lifestyle, and it keeps getting lower as the biofilm matures.

Cross-regulation of Assembly Between Two Components of a Copolymer Determines Bacterial Cell Curvature
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Many bacterial proteins perform functions that require them to properly assemble into higher order structures. Often this assembly process depends on energy sources, as is the case for most cytoskeletal elements in the cytoplasm. Recently, we discovered that cytoskeleton-like filaments can also assemble in the periplasm of Gram-negative bacteria, which lacks ATP or GTP. Specifically, two proteins from Vibrio cholerae, CrvA and CrvB promote cell curvature by assembling into a long filamentous structure in the periplasm. CrvA and CrvB regulate one another’s levels and assembly to form a copolymer. This CrvAB structure localizes along the inner curved face of the bacteria and upon heterologous expression is sufficient to curve all Gram-negative bacterial species tested. Thus, CrvAB appear to form an autonomous periskeletal module sufficient for curvature induction. We have thus taken a multidisciplinary approach to better understand how CrvA and CrvB interact and regulate one another.
Interestingly, CrvB is homologous to CrvA at its N-terminus but has an additional large CrvB-specific domain at the C-terminus. Cells lacking either crvA or crvB exhibit a straight-rod cell morphology and differ in the molecular assembly of the remaining gene product. CrvA assembly is impaired in cells lacking crvB, while CrvB assemblies are absent upon loss of crvA. To understand the molecular and structural basis of these interactions we have purified CrvA and CrvB and examined their assembly in vitro alone and in combination. Preliminary biochemical and electron microscopy studies suggest a new copolymer assembly model. High-resolution structural analysis by cryo-EM is underway to test this model. Here I will describe these findings and their implications for the general processes of energy-independent filament assembly and its role in bacterial cell shape determination.

P901

The evolution of bacterial shape complexity by a curvature-inducing module

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Bacteria can achieve a staggering diversity of cell shapes that promote critical functions like motility, colonization, and virulence. The cell wall defines bacterial shape and is primarily built by two cytoskeleton-guided synthesis machines. Previous studies suggested that complex bacterial shapes evolve by modulating these core machines. In contrast, we discovered that two proteins from *Vibrio cholerae*, CrvA and CrvB, are sufficient to induce complex cell shape autonomously of the cytoskeleton and thus represent a previously-undescribed class of shape determinants. CrvA and CrvB induce curvature by regulating one another to form an asymmetrically-localized periplasmic structure whose assembly drives curvature dynamics. Evolutionarily, CrvA and CrvB appear to have diverged from a single hybrid protein and their specialization promotes rapid curvature induction. The CrvAB module is functional in multiple heterologous species spanning 2.5 billion years of evolution, demonstrating that the evolution of morphological complexity need not involve co-evolution of core cell shape regulation.

P902

No membrane, no problem: condensing bacterial organelles

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Living cells are divided into functional compartments called organelles. In eukaryotes, lipid membranes separate organelles from the cytoplasm such that each compartment maintains a distinct biochemical composition that is tailored to its function. In contrast, prokaryotes typically lack internal membranes and instead must use other mechanisms to spatially organize the cell. Using fluorescence imaging and single-molecule tracking, we show that *E. coli* RNA polymerase (RNAP) organizes into clusters through liquid-liquid phase separation (LLPS). RNAP clusters, or "condensates", increase cell survival during stress, and appear to regulate ribosome biogenesis in response to nutrient availability. Our results demonstrate that bacteria, like eukaryotic cells, use LLPS to generate membraneless organelles that spatially organize biochemical processes to optimize cell fitness in various environments.
A Protease-mediated Switch Regulates the Growth of Magnetosome Organelles

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Many bacteria are known to produce cytoplasmic membrane-bounded compartments. Yet, the mechanisms controlling their biogenesis remain largely unexplored. We use the magnetosomes of magnetotactic bacteria as a model for studying bacterial compartmentalization. Magnetosomes direct the biomineralization of magnetic nanoparticles which, once arranged into chains within the cell, help magnetotactic bacteria navigate along geomagnetic fields. Magnetosome membranes are not uniform in size, and can grow in a biomineralization-dependent manner [1]. However, the underlying mechanisms of magnetosome membrane growth are still unknown. Magnetosome production is genetically controlled by factors including the magnetosome-associated membrane (Mam) proteins, which are primary candidates for testing their involvement of magnetosome membrane growth. Here, using cryo-electron tomography, we find that the protease activity of MamE is required for magnetosome membrane growth. MamE, an HtrA family serine protease, is conserved in all magnetotactic bacteria species and has been previously shown to regulate biomineralization of magnetic particles [2]. Consistent with this finding, the MamE protease activator MamO [3] is also necessary for magnetosome membrane growth. Additionally, we find a new potential MamE protease activator, MamM, which is also required for magnetosome membrane growth. Altogether, these results suggest that the proper regulation of MamE protease activity is critical for magnetosome membrane growth. Furthermore, we used a proteomics approach to find a new MamE proteolytic target, MamD. A non-cleavable MamD mutant inhabits magnetosome membrane growth, indicating MamD needs to be cleaved by MamE for membrane growth. Hence, the growth of magnetosome membrane is controlled by the conserved HtrA protease MamE mediated switch.[1] Cornejo E, Subramanian P, Li Z, Jensen GJ, Komeili A (2016). Dynamic Remodeling of the Magnetosome Membrane Is Triggered by the Initiation of Biomineralization. MBio [2] Hershey, DM, Browne PJ, Iavarone AT, Teyra J, Lee EH, Sidhu SS, Komeili A. Magnetite Biomineralization in Magnetospirillum magneticum Is Regulated by Switch-like Behavior in the HtrA Protease MamE (2016). J Biol Chem. [3] Hershey DM, Ren X, Melnyk RA, Browne PJ, Ozyamak E, Jones SR, Chang MC, Hurley JH, Komeili A. MamO Is a Repurposed Serine Protease that Promotes Magnetite Biomineralization through Direct Transition Metal Binding in Magnetotactic Bacteria (2016). PLoS Biol.

Functional analysis of Ralstonia solanacearum effector, RipAA whose function is regulated by N-terminus phosphorylation in yeast

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Bacterial pathogens employ a type three secretion system to deliver effector proteins into the host cell to disrupt the host defense response. We are investigating the molecular function of effectors from the plant pathogen, Ralstonia solanacearum using the yeast expression system. RipAA is one of the effectors that cause yeast growth inhibition and GFP-tagged RipAA is specifically localized to the daughter cell membrane in yeast cells. Western blot analysis revealed that RipAA is hyperphosphorylated in its predicted auto-inhibitory domain and this phosphorylation is dependent on the plasma membrane targeting in yeast. We found that phosphorylation of RipAA is dependent on yeast casein kinase I (Yck2),
which is localized to the plasma membrane. To clarify the role of phosphorylation in RipAA, we generated a mutant (RipAA<sup>12SA</sup>) that 12 serine/threonine residues of its predicted auto-inhibitory domain were substituted to alanine residues and examined the relationship the growth inhibitory activity and phosphorylation level in yeast. We found that RipAA<sup>12SA</sup> exhibited a loss of growth inhibition and a significant decrease in phosphorylation level in yeast. We also showed that a bacterial expressed recombinant Yck2 phosphorylates the GST-RipAA auto-inhibitory domain fusion protein in vitro. These results demonstrate that RipAA is activated by N-terminus phosphorylation, which might be also important for RipAA function in both yeast and plant.

**P905**

*Dispersing Pseudomonas aeruginosa from surfaces via a self-produced small molecule*


*Pseudomonas aeruginosa* is a significant threat in both healthcare and industrial biofouling. Surface attachment of *P. aeruginosa* is particularly problematic as surface association induces virulence. There are no known factors that specifically disperse surface-attached *P. aeruginosa* and such a factor could be a powerful therapeutic agent. I combined cell biology and natural product chemistry to uncover a potential way to combat a pathogen with its own natural product. I developed a quantitative surface-dispersal assay and use it to show that *P. aeruginosa* itself produces factors that can stimulate its dispersal. Through bioactivity-guided fractionation and chemical analysis, I elucidated the structure of one such factor, 2-methyl-4-hydroxyquinoline (MHQ). Pure MHQ is sufficient to disperse *P. aeruginosa* on its own. Like other alkyl-quinolones, biosynthesis of MHQ in *P. aeruginosa* requires the function of the *pqsABCDE* operon. In addition, MHQ inhibits the dynamic activity of Type IV Pili (TFP) and that TFP targeting can explain the dispersal activity of MHQ. My work thus identifies surface dispersal as a new activity of *P. aeruginosa*-produced small molecules, characterizes MHQ as a promising dispersal agent, and establishes TFP inhibition as a viable mechanism for *P. aeruginosa* dispersal.

**P906**

*Phenotypic memory during feast and famine transitions in bacteria*

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Bacteria in nature spend most of their time in starved states waiting for new resources to appear. Although feast-and-famine dynamics are a fundamental aspect of bacterial life, little is known about how cell physiology changes during starvation and how these changes affect subsequent growth resumption. Here, we address these questions using a novel microfluidic device that allows direct observation of single cells of *Escherichia coli* resuming growth after starvation. Using this device we ask whether bacteria can remember the conditions that they encountered before becoming starved and whether this memory can help them thrive during feast-and-famine regimes. To do this, we study whether starved bacteria resuming growth in lactose benefit from having encountered lactose before
entering starvation and how this benefit varies depending on the duration of starvation. The latter underlies the more fundamental question of whether bacteria ‘forget’ the conditions that they experience before starvation if this period lasts very long. We find that when starvation is short, growth resumption in lactose is faster if bacteria were consuming lactose when becoming starved. This happens because bacteria maintain the machinery for lactose metabolism throughout starvation benefiting from this phenotypic memory to resume growth when lactose appears. Strikingly, we find that as the duration of starvation increases, this pattern shifts and most cells that were feeding on lactose before starvation take longer to resume growth in lactose than cells that were feeding on glucose. Using additional experiments in combination with a model of the intracellular dynamics of lac proteins, we show that this pattern can result from a decoupling between lactose transport and metabolism that occurs because LacY and LacZ proteins likely degrade at different rates during starvation. Overall, our results show that despite growth arrest cells are dynamically changing during starvation in a complex manner that depends on how they entered starvation, is highly individual and influences how they resume growth when resources become available.

P907

Unveiling the intriguing antilisterial mode of action of class Ila bacteriocins
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Few studies have demonstrated that the mode of action of class Ila bacteriocins relies on the recognition and binding of the peptide to the mannose-phosphotransferase (Man-PTS) system of Listeria monocytogenes, specifically to the subunit IIC of the carbohydrate-specific protein complex (enzyme II, EI). Further in vitro assays are warranted to substantiate the proposed mechanism in order to provide a thorough knowledge on this intriguing mode of action, which remains largely uncharacterized. With the ultimate goal of evaluating the specific interaction between pediocin PA-1, a representative of the class Ila bacteriocins, and an extracellular loop of the subunit IIC (loop IIC) of the EI permease of the Man-PTS system of the Listeria strain EGDe (serotype 1/2a), we performed an in vitro binding analysis which has not been previously conducted. Concerning this purpose, a high-throughput screening (HTS) approach was performed in order to select the most suitable pCoofy vectors and Escherichia coli host strains, aimed at expressing the soluble peptides. The proteins were purified, the oligomeric state was investigated, and the affinity of the two ligands through size exclusion chromatography (SEC) was evaluated. The extracellular loop IIC unraveled to be highly insoluble following heterologous expression owing to the fact that the peptide is encompassed in a membrane receptor. In an attempt to circumvent the protein misfolding issue, the HTS identified the pCoofy 16 vector comprising an N-terminal His10-NusA tag, expressed in E. coli C41(DE3) at 20 °C, as the optimum expression conditions. The purification of the soluble neo-synthetized peptide in fusion with NusA was performed through the combined procedure of immobilized metal affinity, and ion exchange chromatography, in which two buffer additives, namely glycerol and β-mercaptoethanol, were utilized to prevent the protein aggregation. An approach based on SEC binding assays, revealed that the recombinant loop IIC per se originated a single peak corresponding to the expected molecular weight of the monomeric recombinant protein (ca. 59kDa), whilst once mixed with the pediocin PA-1 (4.6 kDa) in an equimolar ratio was eluted at a molecular weight of 64 kDa consistent with the formation of a stable complex. Given the observed shift in the retention volume of the refolded NusA-fused loop IIC associated...
to the native pediocin PA-1, one may speculate that the two proteins interacted. To the best of our knowledge, this is the first study documenting a preliminary qualitative evaluation of the interaction of the bacteriocin pediocin PA-1, with the extracellular loop of the membrane subunit IIC of L. monocytogenes. A detailed analysis of the thermodynamic parameters governing this interaction will ensue.

**Regulation of Actin Dynamics and Myosin-Mediated Contraction in Migration**

**P908**

*Nance-Horan Syndrome-like 1 protein negatively regulates Scar/WAVE-Arp2/3 activity and inhibits lamellipodia stability and cell migration.*


Cell migration is important for development and its aberrant regulation contributes to many diseases. The Scar/WAVE complex is essential for Arp2/3 mediated lamellipodia formation during mesenchymal cell migration and several coinciding signals activate it. However, so far, no direct negative regulators are known. We have identified Nance-Horan Syndrome-like 1 protein (NHSL1) as a novel, direct binding partner of the Scar/WAVE complex, which co-localise at protruding lamellipodia. This interaction is mediated by the Abi SH3 domain and two binding sites in NHSL1. Furthermore, active Rac binds to NHSL1 at two regions that mediate leading edge targeting of NHSL1 suggesting that Rac recruits NHSL1. Surprisingly, NHSL1 inhibits cell migration through its interaction with the Scar/WAVE complex. Mechanistically, NHSL1 may reduce cell migration efficiency by impeding Arp2/3 activity, as measured in cells using a novel Arp2/3 FRET-FLIM biosensor, resulting in reduced F-actin content of lamellipodia, and consequently impairing the stability of lamellipodia protrusions.

**P909**

*TRPV4/CaM-dependent protein kinase II/RhoA Calcium Signaling Axis Facilitates Cell Migration by Reinforcing Actin Network in Lamellipodia*

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Cell migration underlies a wide range of physiological processes from embryonic development to tissue regeneration, and its deregulation is a key factor in numerous pathologies including cancer. Several signaling networks including small GTPases and Ca^{2+}-dependent signaling pathways have been identified as master regulators of cell migration. These networks control the organization and dynamics of the actin cytoskeleton, allowing cells to form lamellipodia, an actin-based ‘engine’ that drives the cell forward. Although it is well understood how small GTPases regulate the cytoskeleton, the role of Ca^{2+} signaling in lamellipodia assembly remains elusive. By using cell spreading assay, we screened a panel of siRNAs targeting mechano-gated ion channels and identified a single Ca^{2+} channel TRPV4 to be essential for lamellipodia protrusion. Analysis of Ca^{2+} dynamics revealed a transient increase of Ca^{2+} level in protruding lamellipodia that was abolished upon TRPV4 inhibition. Furthermore, inhibiting TRPV4 decreased the density of filamentous actin (F-actin) in lamellipodia, indicating that formation of the
lamellipodial actin network is fostered by Ca²⁺ influx through TRPV4. To understand how TRPV4-mediated Ca²⁺ signaling regulates the structure and dynamics of actin cytoskeleton, we interrogated the involvement of Rho family GTPases, the master regulators of actin dynamics, by using FRET biosensors. We found an enrichment of RhoA activity in protruding lamellipodia of the control cells that coincided spatially with the Ca²⁺ influx. Surprisingly, such local upregulation of RhoA activity was dampened upon TRPV4 inhibition, suggesting that TRPV4-mediated Ca²⁺ signaling sustains RhoA activity in lamellipodia. By immunostaining we showed that suppression of TRPV4 decreased the amount of active Ca²⁺-calmodulin-dependent protein kinase II (CaMKII), a key Ca²⁺ signaling hub, in lamellipodia. Furthermore, we showed that local inhibition of CaMKII halted cell protrusions, suppressed RhoA activity and decreased F-actin density in lamellipodia, suggesting that CaMKII transduces local Ca²⁺ signals from TRPV4 to RhoA. By using phosphoproteomic profiling, we identified RhoA regulatory proteins that are phosphorylated in a TRPV4- and CaMKII-dependent manner and thus, may act as a molecular link between Ca²⁺ and RhoA signaling pathways in lamellipodia. Finally, we showed that suppression of this new signaling axis suppresses cell protrusive activity and migration in a tissue-mimicking microenvironment. Together these data elucidate a novel Ca²⁺ signaling axis composed of TRPV4/CaMKII/RhoA that reinforces lamellipodial actin network, facilitates lamellipodial protrusions and promotes cell migration.

P910

Shootin1b-mediated leading process extension triggers Ca²⁺ transient for somal translocation during neuronal migration
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Neuronal migration is a fundamental process to establish brain architectures; impaired neuronal migration leads to brain agenesis and diseases. As a unique feature of their migration, neurons move in a saltatory manner by repeating leading process extension and somal translocation. Therefore, neurons must coordinate these two steps to migrate properly. Previous studies reported that transient increases in intracellular Ca²⁺ concentration, which is called Ca²⁺ transient, positively correlate with somal translocation of cerebellar granule cells (Komuro and Rakic, Neuron, 1996; Kumada and Komuro, PNAS, 2004). In addition, another study reported that Ca²⁺ transient is involved in the activation of actomyosin to drive somal translocation (Martini et al, J Neurosci, 2010). These finding suggest that the cell signaling via Ca²⁺ transient drives somal translocation. However, the mechanism which triggers Ca²⁺ transient for somal translocation remains unclear. Here, we found that the length of leading process positively correlates with the frequency of Ca²⁺ transient. We recently reported that shootin1b is a key molecule to drive leading process extension; its gene knockout results in the inhibition of leading process extension of olfactory interneurons (Minegishi et al, Cell Rep, 2018). Our Ca²⁺ imaging analysis showed that the frequencies of Ca²⁺ transient and somal translocation were decreased in shootin1 KO olfactory interneurons. These findings raise a possibility that shootin1b-mediated leading process extension may trigger Ca²⁺ transient for somal translocation during migration of olfactory interneurons.
P911

Cell-cell signaling through Fat2 restricts WAVE complex-dependent protrusions to the leading edge
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During crawling cell motility, cells build protrusions at their leading edge and retract at their trailing edge to move forward. Cells migrating as a collective need to ensure that their leading and trailing edges align with those of their neighbors, a form of planar cell polarity. Here, we investigate how the cells of the Drosophila egg chamber epithelium align their leading edges with their neighbors during one such collective migration. The follicle cells that make up the egg chamber epithelium undergo a highly coordinated collective migration along an underlying basement membrane extracellular matrix. The tissue lacks leader cells or a free edge, and tissue-level polarity emerges from cell-cell interactions across the field. We show that Fat2, an atypical cadherin that localizes to the trailing edge, promotes lamellipodial protrusion by concentrating the WAVE regulatory complex in clusters at the leading edge of the cell behind. Without Fat2, the WAVE complex distributes around the cell edge, causing protrusions to form in randomized, unaligned directions and collective migration to fail. This local planar signaling mechanism is well-suited to direct collective migration in an edgeless epithelium where there is no global cue to which the cells can align.

P912

Pikfyve plays a positive regulatory role in cell migration via regulation of lamellipodia dynamics
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Cell migration is a critical process in development and its misregulation is associated with cancer metastasis. PIKfyve, the sole lipid kinase responsible for the synthesis of phosphatidylinositol 3,5-bisphosphate (PI3,5P₂) is essential for several endosomal and lysosomal functions. Studies have suggested that PIKfyve plays a role in cell migration, but the localization of PIKfyve and the molecular mechanisms whereby PIKfyve regulates cell movement remained to be established. Importantly, in our unpublished studies (abstract #2002), we found that endogenous PIKfyve localizes to endosomes that are active in recycling, and that PIKfyve plays a direct role in integrin recycling. These studies suggest that PIKfyve-dependent recycling of integrins partly accounts for a role for PIKfyve in cell migration. Here we uncover two additional mechanisms, whereby PIKfyve regulates cell migration. We found that a pool of endogenous PIKfyve also localizes to the plasma membrane at the cell leading edge. Notably, using live-cell imaging we discovered that acute inhibition of PIKfyve, over a period of 5-10 minutes, significantly impairs the dynamics of lamellipodia. Moreover, using a fluorescence resonance energy transfer (FRET)-based sensor during that same time-frame, we found that PIKfyve is required for the activation of Rac1 at the cell periphery. These results suggest that PIKfyve localization to the cell leading edge stimulates Rac1-mediated lamellipodia dynamics, crucial for cell migration. Furthermore, we discovered that PIKfyve inhibition decreases the localization of both Kindlin2 and the Arp2/3 complex in lamellipodia. Notably, previous studies showed that Kindlin2, a scaffold protein that interacts with Arp2/3 complex, binds directly to PI3,5P₂ in vitro. Together, these discoveries suggest that PIKfyve has two roles at the plasma membrane in the regulation of lamellipodia dynamics: 1) PIKfyve is required for Kindlin2 localization, which likely accounts in part for how PIKfyve recruits the Arp2/3 complex, and 2)
PIKfyve is required for activation of Rac1, which also likely contributes to the PIKfyve-dependent recruitment of the Arp2/3 complex. These new findings, combined with our findings that PIKfyve plays a direct role in the recycling of integrins, suggest that PIKfyve controls several pathways in cell migration. Thus, PIKfyve may provide a higher order regulation of the cell migration machinery.

P913

Feedback and polarity in directed cell migration
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The ability of eukaryotic cells to move in specific directions towards signals is critical for development, immune response, and disease. In order to produce forward motion, cells must coordinate pushing forces from polymerization of cytoskeletal actin at the cell front and activity of the actin-binding motor Myosin, which mediates contraction at the cell back. To organize these forces, cells utilize a complex, interconnected signal transduction network which activates or inhibits actin polymerization and Myosin activity in different cellular regions. In the presence of an extracellular cue like an antigen or force, this same network can persistently orient the cell front towards the signal. Many cells can also polarize these activities, creating semi-permanent fronts or backs with greater or lesser activities. Molecules involved in signal transduction and polarization, like Ras family small GTPases, are also highly mutated in cancers but have proved difficult to reliably inhibit. Signal transduction, the cytoskeleton, and polarization are linked together through feedback loops, leading to complex interactions that make the assessment of individual molecules difficult. Therefore, understanding how different molecules in this pathway affect the behavior of the network as a whole is vital to understanding how cells are able to move processively. Using tools to change cell mechanics and cytoskeletal organization in real time, we analyze how feedback from the cytoskeleton onto signaling networks controls cell migration and cell polarity in eukaryotic cells.

Role of Lipids in Stress Response and Organelle Architecture

P914

Acute manipulation of outer membrane phospholipid composition directly alters mitochondrial dynamics and ultrastructure.

Mitochondria continuously undergo coordinated rounds of fusion and fission that are critical for maintaining the functional integrity of this essential organelle. Altered mitochondrial dynamics are now known to be associated with several prominent human diseases and numerous protein effectors have been established as key regulators of mitochondrial morphology. However, the importance of the local lipid composition during mitochondrial fusion and fission processes has received much less attention. As a result, we devised a chemically-inducible dimerization system using a modified phospholipase C from bacteria (bacPLC) to initiate the localized hydrolysis of the core structural phospholipid, phosphatidylinositol, selectively within the cytosolic leaflet of the outer mitochondrial membrane.
POSTER ABSTRACTS

(OMM). Using this unique tool, we show that recruitment of bacPLC to the OMM not only causes the expected local accumulation of the direct enzymatic product, diacylglycerol (DG), but also initiates the rapid and uniform fragmentation of the mitochondrial network. Mitochondrial fission induced by bacPLC is accompanied by profound swelling of the mitochondrial matrix along with notable ultrastructural changes, including marked vesiculation of the inner mitochondrial membrane (IMM) and a general loss of cristae. These dramatic morphological changes occur within minutes of tethering bacPLC to the OMM and do not result in alterations to the OMM potential or matrix Ca\(^{2+}\) concentration, which suggests that the changes observed are not due to gross insults to the integrity of the OMM. We then examined the impact of acutely changing OMM lipid composition on the regulation of established components of mitochondrial fission machinery, focusing specifically on the ubiquitously-expressed GTPases dynamin-related protein 1 (Drp1) and dynamin 2 (Dnm2). Initial studies using dominant-negative constructs suggest that both Drp1 and Dnm2 work in concert to drive efficient bacPLC-induced mitochondrial fission. However, results using an established Drp1 knockout cell line show that the loss of Drp1 alone is sufficient to prevent the mitochondrial fragmentation initiated by bacPLC and, therefore, Drp1 is likely to function upstream of Dnm2 recruitment in this context. Interestingly, unlike during mitochondrial fission, the inhibition or removal of Drp1 does not prevent the prominent mitochondrial swelling that is observed in response to the acute generation of DG within the OMM by bacPLC. Overall, these studies establish a direct relationship between lipid metabolism within the OMM and clinically-relevant morphological changes that are known to manifest in mitochondrial-associated diseases, including molecular events that function to coordinate the remodeling of both the IMM and OMM.

P915

**Endoplasmic reticulum responding to compressive stress**


The endoplasmic reticulum (ER), responsible for synthesis and export of all secretory and membrane proteins, is the cell’s largest organelle which makes contact with nearly every other part of the cell. Its structure is composed of dynamic tubules and sheets that create an intricate network through which protein synthesis, translocation, and secretion can occur. This vast, flexible network is dynamic and constantly remodeling itself, suggesting an acute sensitivity to stimuli such as compressive stress or altered cellular rheology. Here we present morphological and dynamic changes to the ER and protein secretory pathway in response to mechanical stress. Through live-cell compression experiments and fluorescence microscopy, we characterize the ER response to physical, compressive stress. Together with in vivo observations of cargo trafficking through the ER, these findings suggest that aspects of the ER, including its structural organization and biochemical makeup, are targets of physical deformation. These observations lead us to hypothesize that environments of increased physical stress, such as the dense tissue of a tumor, impede regular function of the protein secretory pathway.

P916

**Genome-wide CRISPR-Cas9 knockout screen to dissect ceramide cytotoxicity**

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Ceramides are a class of sphingolipids tightly linked to a variety of pathological conditions. In metabolic tissues, elevated ceramide levels inhibit PI3K and Akt activities, leading to insulin resistance. Aberrant ceramide levels can also induce cell death. It remains poorly characterized how ceramide accumulation impairs cellular functions. In this work, we performed a genome wide CRISPR-Cas9 genetic screen to identify regulators of ceramide-induced cell death in mouse preadipocytes. The screen isolated known mediators of ceramide metabolism such as ceramide synthase (CERS5) but most of the identified genes were not previously linked to ceramide cytotoxicity. We anticipate that these genes regulate ceramide-linked proapoptotic and antiapoptotic pathways. This work may also shed light on other ceramide-linked diseases such as insulin resistance.

P917

**Palmitoylation in the Control of Assembly and Function of a Plasma Membrane PI 4-Kinase Complex**


Phosphoinositides (PIPs) are low abundance phospholipids that play critical roles in eukaryotic membranes. The plasma membrane (PM) is highly enriched in PI(4,5)P₂ and its precursor PI(4)P, which is synthesized by PI 4-kinase Type IIIα (PI4KIIIα). Biochemical evidence suggests PI4KIIIα exists in two multicomponent complexes at the PM. EFR3B, a multiply-palmitoylated inner leaflet protein, acts to anchor the kinase to the membrane in both complexes, but one complex additionally contains a six-pass transmembrane protein, TMEM150A. The functional differences between these complexes remain mysterious, as well as mechanisms regulating the assembly of each complex. To shed light on TMEM150A’s role in PI4KIIIα-mediated PI(4)P production, we investigated its interactions with EFR3B. Using a combination of fluorescence recovery after photobleaching, imaging-based detergent resistance membrane assays, and EFR3B mutants with altered palmitoylation patterns, we found that EFR3B palmitoylation regulates its interaction with TMEM150A, namely that TMEM150A exhibits selectivity for specific palmitoylation patterns of EFR3B. This selective binding contributes to the formation of the TMEM150A-containing PI4KIIIα complex, which is proposed to synthesize a pool of PI(4)P dedicated to rapid conversion to PI(4,5)P₂ after acute depletion, thus maintaining PIP homeostasis at the plasma membrane. These observations suggest that the lipid bilayer itself, via interactions with palmitoylated forms of EFR3 and the transmembrane helices of TMEM150A, may regulate PI(4)P synthesis to ensure proper PIP levels at the plasma membrane.

P918

**Plasma membrane compartment of Can1 modulates sphingolipid biosynthesis in response to stress**

**J. Zahumensky¹**, C. M. Fernandes², M. Del Poeta², J. B. Konopka², J. Malinsky³; ¹Institute of Experimental Medicine of the Czech Academy of Sciences, Prague, CZECH REPUBLIC, ²Department of Microbiology and Immunology, Stony Brook University, Stony Brook, NY.

Fungal infections caused by *Candida albicans* claim the lives of hundreds of thousands each year. The most common antifungal treatment relies on the use ofazole drugs targeting biosynthesis of ergosterol. However, the constant emergence of new resistant strains clearly shows that alternative strategies are desperately needed if we are to combat fungal infections effectively. One such strategy could make use of the MCC (membrane compartment of the arginine permease Can1) microdomain, which was
originally identified in the yeast *Saccharomyces cerevisiae* and was shown to be essential for hyphal growth and virulence of *C. albicans*. Several proteins accumulating at the MCC, such as Nce102, are involved in the regulation of biosynthesis of sphingolipids, a vital structural component of the eukaryotic plasma membrane. Following inhibition of sphingolipid biosynthesis by myriocin, the plasma membrane pattern of Nce102-GFP loses its typical patchy character and becomes homogeneous, whereupon the whole MCC destabilizes, leading to activation of TORC2 and in turn sphingolipid biosynthesis. Due to this apparent movement of Nce102, the protein has been proposed to be a sphingolipid sensor. A decade after this discovery, the molecular mechanisms of the sensing have not been entirely elucidated. In the present study we show that the redistribution of Nce102 in response to myriocin treatment is accompanied by elevation of the cellular amount of the protein. To demonstrate that these changes are not limited to direct inhibition of sphingolipid biosynthesis, we selected a range of chronic stress conditions targeting either the lipid composition or integrity of the plasma membrane. While changes in Nce102 localization in the model yeast *S. cerevisiae* are quite subtle, effects of the stress on *C. albicans* are readily observable. Similarly, elevation of Nce102 amount is greater in the pathogen. In order to ascertain the extent and character of MCC contribution to lipid homeostasis under used stress conditions, we employed mass spectrometry to study sphingolipid composition in the *nce102* deletion strain and compared it to the respective wild type. While sphingolipid biosynthetic pathways in humans and fungi share certain common themes, regulation mechanisms and chemical identity of the final products are distinct in the species, which could be exploited in treatment of fungal infections. Furthermore, our study adds to the mounting evidence that the MCC microdomain is a valuable, fungi-specific, pharmacological target. This project is supported by the Czech Academy of Sciences, grant MSM200391901.

Science Education: Basic Science Research

P919

The DNA Detectives Afterschool Program: A Service-Learning Course that Intersects Informal STEM learning, Youth Mentoring, and Science Communication

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Service Learning is pedagogical practice to enhance student learning through a targeted service that meets a community need. It is a high impact practice that allows students to put their skills into real world practice and then to reflect on the effect of that application on their own learning. While service-learning is widespread in the humanities, it is less utilized in the STEM fields. Here, I describe an upper level service learning course for biology majors in which students facilitated an afterschool outreach program for middle school students in an underserved, urban community. The six week afterschool program was developed in partnership with a faith-based program, Erie City Mission’s Urban University, and focused on both leadership development and informal STEM learning. Undergraduate students served as mentors to youth in the program in a 1:1 ratio and designed activities and curricula around DNA structure and technology. Throughout the course, students reflected upon how the development of course materials and facilitation of activities enhanced their own understanding of the material, laboratory skills, and science communication skills. Students also reflected on the development of science identity and gained novel perspectives on how inequities and exclusion in STEM learning is related to individual and systemic differences in education and resources. Data collected from
reflections, post-programs interviews, and questionnaires suggest that the program provides a robust means to mentor middle school youth, work with faith-based organizations through informal STEM learning, and provide a highly meaningful and application-based learning experience for undergraduate students.

P920

Dartmouth rural STEM educator partnership

R. D. Sloboda1, V. V. May2, M. T. Tine1, A. A. Skinner1, V. L. Foster-Johnson3, L. Brahms4, M. Fenzel4, K. E. Price4; 1Dartmouth College, Hanover, NH, 2Thayer School of Engineering, Hanover, NH, 3Geisel School of Medicine, Hanover, NH, 4Montshire Museum of Science, Norwich, VT.

Educating students from an early age about science, technology, engineering, and mathematics (STEM), and maintaining their engagement with scientific thought and analysis, is crucial for their long-term understanding of science and the world around them. Loss of interest by students not only toward careers in STEM but also learning STEM is a national problem and begins in the middle school years. This problem is particularly acute in low-income rural communities, where there are little, if any, resources and teachers have very few options for professional development and networking. To enhance rural science education, our project engages 6th – 8th grade teachers in low-income rural communities as equal partners with Dartmouth faculty, graduate students, and science educators from the Montshire Museum of Science. Together we are developing a series of Next Generation Science Standards-aligned, hands-on, investigative, crosscutting curricular units to enhance middle school science education. During the first year of the project, we developed a month-long teaching unit on climate change, using ticks as the focal point. The unit includes extensive lesson plans, multi-media content, hands-on activities, and a full package of materials and supplies for each teacher. The project has three sections, and each section is introduced via a video showing an evening newscast in which the reporters present data about various changes local residents are detecting relative to organisms and the environment. Each newscast—presented by graduate students acting as reporters who later serve as near peer mentors in the classroom—forms the basis for a series of observations, hands-on experiments, and discussions that engage young scientists directly in the act of discovery, data analysis, and presentation. Our first unit has been developed, but not yet tested extensively in a classroom setting in our four pilot schools due to the current pandemic. Once tested and evaluated, the content of the unit will be revised as necessary and then made available via the project website [https://sepa.host.dartmouth.edu/] to other schools in NH and VT and elsewhere. This unit is targeted to the sixth grade. Subsequent years of this project will develop similar units for seventh and eighth grade science classes.

P921

Extracurricular Biology Research in a High School Setting


Students at Sidwell Friends School in Washington, D.C. have the opportunity to conduct original research both inside and outside of the classroom. Students who want to further pursue molecular biology research join the Biological Research and Investigations in Neuroscience (BRAIN) Club and often
partner with scientists from research institutions. Extracurricular research enables students to employ research techniques they have gained through science classes in lab-like settings. This year, students chose to conduct experiments on Danio rerio, or zebrafish. Their projects include: (i) Testing the effects of ethanol exposure on the motor system of fully grown zebrafish, (ii) Evaluating the physiological and morphological effects of mutations of the col5a1 gene in zebrafish embryos, (iii) Comparing the effects of aspartame and household sugar on the development of zebrafish embryos, and (iv) Comparing the effects of nicotine and e-cigarette fluid on developing zebrafish embryos.

P922

Introductory Biology Research in a High School Setting

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Students at Sidwell Friends School in Washington, D.C. have the opportunity to conduct original research both inside and outside of the classroom. Students who want to further pursue molecular biology research join the Biological Research and Investigations in Neuroscience (BRAIN) Club and often partner with scientists from research institutions. Extracurricular research enables students to employ research techniques they have gained through science classes in lab-like settings. This year, students chose to conduct experiments on Danio rerio, or zebrafish. Their projects include: (i) Testing the morphological effect of varying concentrations of nitrites and nitrates on zebrafish embryos, (ii) Testing the effects of the plastic contaminant polyethylene terephthalate (PET) on zebrafish embryos, (iii) Testing the effects of the pharmaceutical contraceptive ethinylestradiol on Danio rerio embryos, and (iv) Observing the effects of RoundUp on the nervous and motor system of zebrafish.

P923

Incorporating open data from the Allen Institute for Cell Science into biology classrooms

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As part of our mission to support open science, the Allen Institute freely shares all of the data we generate for anyone to use in research or teaching. While these resources are developed primarily for researchers, many educators have incorporated data, tools, and information available on allencell.org into their classrooms over the years and we have since begun working with select educators to develop resources specific for use in education. These fully online resources enable students to conduct virtual experiments that are particularly well suited for distance learning during the pandemic. The Allen Institute for Cell Science uses diverse technologies and approaches at a large scale to study cells and their components as integrated systems. Our live imaging data of the major cell structures, fluorescently tagged by genome-editing human induced pluripotent stem cells, is used to develop predictive models of cell states and behavior. We provide numerous tools to make it easier for researchers and students to visualize 3D volumes and to study quantitative measurements of cellular structures directly in a web browser. Resources specifically developed for educators include formal virtual labs published in CourseSource (Shelden et al., 2019; Goller et al., submitted); webinars for educators demonstrating how to run the virtual labs; and presentations from teachers describing how they use resources such as the
Visual Guide to Human Cells web application, which can be used to introduce students to cellular anatomy and function using models derived directly from whole cell data that accurately portray the 3D nature of cells. Data resources developed for research use that are particularly popular with educators include the 3D Cell Viewer and Cell Feature Explorer, which provide students with access to thousands of 3D cell images and tools to conduct quantitative analyses directly in their browser, as well as image datasets for cell colonies that have been perturbed by various drugs, which can be downloaded and analyzed with open-source tools for more advanced projects. The quantity and quality of data available through allencell.org enables fully online lab work or projects in combination with data students collect in lab themselves for comparative analysis. Projects range from short virtual lab exercises, such as describing the frequency of mitotic events online, to sophisticated upper level college projects that derive hypotheses and perform segmentation with downstream analyses performed on the huge collection of 3D images available.

P924

The problem-solving process of experts, characterized as decisions-to-be-made: implications for biology education

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A primary goal of science education is to produce good problem solvers, but this has been difficult to teach effectively because much of the problem-solving process remains poorly understood. To characterize this process in terms of measurable and teachable pieces, we analyzed how experts across scientific and engineering disciplines solved authentic problems. We interviewed over 50 faculty and senior researchers about their problem solving, asking them to describe problems they’d solved as part of their professional work - these ranged from troubleshooting techniques to entire research projects. We then analyzed the interviews to identify decisions they needed to make during the process. We identified a set of 29 decisions-to-be-made that were unexpectedly consistent across science, engineering, and medical fields. These decisions fall into 6 categories. 1) Selection and goals, which includes deciding whether a problem is important and deciding on the goals and criteria for a solution. 2) Frame the problem, which includes identifying important features, narrowing the problem down to specific questions or hypotheses, and identifying potential solutions to pursue. 3) Plan process for solving, which includes making needed assumptions and decomposing the problem, deciding and prioritizing what information is needed, and making specific experimental plans for obtaining information. 4) Interpret information and choose solutions, which includes deciding how to analyze and organize data and deciding what are appropriate conclusions. 5) Reflect, which includes reflecting on the choices made in other decisions, on the overall solving approach, and on the final solution. 6) Implications and communication, which includes deciding the best way to present the work. We also find that the process of making these decisions (selecting between alternative actions) relies heavily on predictive models that embody the relevant specialized disciplinary knowledge and standards. This detailed characterization of problem solving will allow better teaching and assessment of problem solving in science. We propose that to learn to become expert-like problem solvers, students need to “deliberately practice” making each decision, while receiving timely feedback on their choices. This means that students must be given problems that require them to engage in these decisions. In this presentation, we will discuss the development of our problem-solving decisions framework with examples from biology. We will also describe the development of a biology problem that engages
students in a large number of these decisions. This problem can be used as a tool for student learning and as an assessment to measure how well a course or program is preparing students to become expert problem solvers.

P925

**Exploring Christianity as a Concealable Stigmatized Identity (CSI) in graduate biology programs**

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A current thrust in biology education research is aimed at increasing participation of underrepresented groups in biology. Prior research has focused on women and students of color, partially because they have been marginalized in society, but one large group of students that have not been historically marginalized in society but are underrepresented in biology are Christians. Although Christianity is the cultural norm in the American public, Christians in academic biology are in the minority and have been stereotyped as less competent than other groups in science. However, Christian biologists could be important boundary spanners for communicating science to the public, most of which identify as Christian. To characterize experiences that Christian students have that could contribute to their underrepresentation in academic biology, we explored Christianity as a stigmatized identity in biology among graduate biology students. We created interview questions based on constructs from a prior framework used in social psychology to study experiences of stigma - the Concealable Stigmatized Identities (CSI) framework - and conducted semi-structured interviews with 33 Christian graduate students in biology programs at 16 research institutions. We asked the Christian graduate students to describe how Christians are perceived within the biology community and their experiences revealing and/or concealing their Christianity in the biology community. We analyzed the interviews using both deductive and inductive coding to find themes across interviews. We found that Christian graduate students perceived that Christians are negatively stereotyped as unintelligent, unaccepting of science, and extremists and/or fundamentalists in the biology community. Christian students were afraid that those in the biology community would stereotype them as less competent scientists, bigoted, or intolerant if they were to reveal that they are Christian. Most students reported that Christians are made fun of in the biology community or that people in biology community responded negatively when the student revealed their Christianity. Many students described having to reveal their identity to correct negative stereotypes about Christians in the biology community. Students often said they were not overt about their Christianity, but did not actively hide it, while other students described being actively covert about their identity, particularly around anti-religious individuals or senior graduate students or professors. These results indicate that Christianity is a Concealable stigmatized Identity (CSI) in biology graduate programs.

P926

**Educating Biomedical Students on Rigor and Reproducibility in Scientific Research: NIDDK Information Network (dkNET) Summer of Data Student Internship Program**

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The NIDDK Information Network (dkNET; https://dknet.org) is an open community resource portal for researchers supported by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). dkNET has developed tools, resources, and training modules to enhance the awareness about scientific rigor and reproducibility. dkNET assists researchers in finding and evaluating research resources such as antibodies, organisms, plasmids, cell lines, biosamples, data, software tools for use in their research. Resource Reports provide researchers aggregated resource information, alerts when resources have problems, and validation information. The Discovery Portal connects researchers to over 200 biomedical databases and hundreds of millions of data records. dkNET also provides information and tools to help researchers comply with NIH mandates for data sharing and rigor/reproducibility by providing an authentication report and information on data management plans and FAIR (Findable, Accessible, Interoperable, Reusable) Data Principles. Through the Hypothesis Center, partnering with the Signal Pathways Project (SPP) knowledgebase, dkNET provides a FAIR omics meta-analysis platform for researchers to model signaling events and generate hypotheses. This year, dkNET launched the 2nd year of the dkNET Summer of Data Student Internship Program, training students on best practices to enhance rigor and reproducibility and the basics of good data management by following the FAIR data principles. Based on the feedback from the prior year, the program was altered to include a one-week bootcamp, an increase in total hours, and added weekly hands-on sessions for in-depth learning and discussion. We used Zoom, Slack, YoTribe, and traditional email for distance learning, group discussion, and communication. Course topics included dkNET, Rigor and Reproducibility, SPP, FAIR Data, Open Science, Data Management, and Protocols.io. After the bootcamp, students utilized dkNET tools and resources in their summer research project. We conducted an anonymous web-based course survey using Qualtrics with open-ended, rating, and Likert scale questions to allow students to provide feedback and help course development. The survey indicated that overall satisfaction with the program was high, and students are confident about their knowledge of these tools and concepts and are comfortable to perform reproducible research after the program. In summary, the program is effective in educating students on scientific rigor and reproducibility and how to apply tools in their research. Biomedical graduate programs or libraries can integrate the dkNET tools, resources, and course materials into their research training curriculum. Source of Support: NIH NIDDK Grant U24DK097771

Secretory Pathway

P927

A physical model of TANGO1-mediated bulky cargo export
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Collagens are the main component of the extracellular matrix, a proteinaceous network that provides the structural integrity necessary for multicellularity. Collagens are bulky secretory proteins, which are de novo synthesized into the endoplasmic reticulum (ER), from where they are exported to the Golgi complex before being secreted. Despite their fundamental importance, the molecular and biophysical mechanisms of how collagens are exported from the ER still remain poorly understood. An ER-resident transmembrane protein, TANGO1, is required for the export of collagens by modulating and physically
connecting the cytosolic COPII membrane-remodelling machinery to the collagens in the ER lumen. We recently monitored by super-resolution nanoscopy the organization of TANGO1 at collagen export sites, showing that TANGO1 assembles into rings around COPII proteins. In this talk, I will present a physical model in which TANGO1 forms a linear filament that wraps around COPII lattices at ER exit sites (ERES) to favor transport intermediate shapes with open necks. Importantly, our results show that ER membrane tension regulation, possibly mediated by TANGO1, can help stabilize such open membrane conduits and thus allow procollagen loading and export from the ER. Altogether, our combined theoretical and experimental findings highlight the crucial role that TANGO1 can play as a key regulator of the mechanical properties of the ER membrane to mediate efficient procollagen export.

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**Cargo Control of Bulky Transporters in TANGO1-Mediated Procollagen Export**

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Collagen is an essential component of the extracellular matrix, constituting 25% of the dry weight in mammalian body. Its precursor, procollagen, is exported from the endoplasmic reticulum (ER) in the form of rigid 300nm long triple-helical structures. However, the mechanisms underlying the biogenesis of bulky transporters for procollagen export remain mostly unknown. Surprisingly, the export machinery for procollagen relies on COPII components, which typically form 80nm spherical carriers, much smaller than procollagen cargos. Recently the ER-resident protein TANGO1 has been shown to organize into rings around COPII coats at ER exit sites (ERES), suggesting it plays a key role in facilitating the formation of large procollagen carriers. Here we propose a continuum biophysical model to study the interplay between procollagen, COPII, and TANGO1 in the formation of a bulky carrier. Our model is based on the Helfrich bending energy of lipid membranes, complemented by a thermodynamic contribution of two interacting membrane-bound species, namely COPII and TANGO1. Using non-linear Onsager’s formalism, we computationally solve the model to predict the coupled dynamics of the lipid membrane and proteins. Our results show that TANGO1 stabilizes incomplete buds by self-organizing into rings around COPII domains, in agreement with experimental observations. We then test three biologically relevant mechanisms hypothesized to contribute to the formation of bulky carriers compatible with procollagen export: (i) membrane tension modulation by recruitment of ER-Golgi intermediate compartments (ERGIC), (ii) force generation by procollagen triple helix folding, and (iii) neck-size control by cargo steric interaction. We show that although each of these mechanisms can independently lead to the formation of large carriers, their coordinated action allows for efficient packing of procollagen. All together our results support a model in which cargo properties and TANGO1 enable the robust formation of bulky procollagen carriers by orchestrating biophysical cues at the ERES.
**P929**

**Prodomains regulate the generation of Nodal signaling during animal development**

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Developmental signaling proteins are secreted asymmetrically in the early animal embryo in order to generate diverse tissues. Nodal and Vg1 (a.k.a. GDF1/GDF3) are TGF-beta-related proteins that form obligate heterodimers to induce mesendoderm formation in animal embryos. In zebrafish, vg1 is maternally deposited and its protein is found in the endoplasmic reticulum (ER) prior to zygotic expression of nodal at the yolk margin. We examine how Vg1 and Nodal prodomains influence Vg1-Nodal signaling. First, we find that glycosylation sites and an exposed cysteine thiol of the Vg1 prodomain mediate its retention in the ER. ER retention of Vg1 may ensure that Vg1 participates in heterodimers with Nodal. Second, by developing a synthetic secreted protease to control cleavage of the Vg1 prodomain, we find that prodomain cleavage is not required for Vg1-Nodal secretion and can occur in a non-cell autonomous manner. Third, while prodomains of Nodal and Vg1 remain associated with secreted heterodimers, only cleavage of the Vg1 prodomain is required for receptor activation in target cells. These findings reveal how Vg1 and Nodal prodomains regulate the generation of functional signaling *in vivo*.

**P930**

**Investigating the energy demand of the secretory pathway in cancer cells**

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The secretion of proteins from the endoplasmic reticulum (ER) via the Golgi apparatus towards the extracellular space is accomplished by the secretory pathway. Cancer cells display particularly high efficiency in this process making it a potential therapeutic target. However, it remains elusive how much energy is needed to maintain key protein trafficking mechanisms within the secretory pathway. Therefore, our aim is to better understand mutual interrelations between the ER-Golgi protein transport and the metabolic activity in HeLa cells, representing a widely used cancer cell model. We have successfully established the high-resolution visualization and analysis of synchronized protein trafficking within the secretory pathway via fluorescent probes in HeLa cells by applying live-cell fluorescence microscopy techniques and semi-automated image-analysis. Fast and efficient protein transport from the ER to the Golgi could be averted by treating HeLa cells with 2-deoxy-D-glucose (2-DG), which induces strong ATP depletion and ER stress. Moreover, we studied the effects of 2-DG on the calcium ion (Ca\(^{2+}\)) concentration in the ER, since ER Ca\(^{2+}\) is known to influence both protein folding and secretion. ER Ca\(^{2+}\)-levels remained unaltered in response to 2-DG, indicating energy stress mechanisms that counteract the ER Ca\(^{2+}\)-leak. In contrast, cytosolic Ca\(^{2+}\)-levels and dynamics displayed massive differences to control conditions. The acquired data from applying fluorescent protein-based probes and advanced imaging techniques hint at yet unknown connections between the secretory pathway, disturbances in energy metabolism and Ca\(^{2+}\)-homeostasis.
P931

Steady-state Regulation of Secretory Cargo Export by Inositol Trisphosphate Receptors and Penta EF Hand Proteins

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In ER-to-Golgi vesicular trafficking, COP II coat assembly is influenced by calcium sensitive proteins, and calcium release from the ER regulates steps of COP II targeting and functionality. For example, sustained agonist induced Ca²⁺ signaling by inositol trisphosphate receptors (IP3Rs) leads to depression of cargo export through penta EF hand (PEF) protein apoptosis-linked gene 2 (ALG-2) which, under these extreme calcium conditions, reduces outer COP II coat at ER exit sites (ERES). In contrast, the regulation of ER export by steady-state Ca²⁺ signaling remains poorly understood. Here, we report that partial depletion of the major ER Ca²⁺ release channel, inositol trisphosphate receptor (IP3R), caused a marked increase in basal ER export of the transmembrane glycoprotein cargo VSV-G in normal rat kidney (NRK) cells. This effect required ALG-2 and was accompanied by accumulation of outer COP II coat and ALG-2 at ERES and loss of Peflin from ERES - a condition previously demonstrated to stimulate COP II-dependent ER export. Increased ER export correlated with a reduction in spontaneous cytosolic Ca²⁺ oscillations most likely caused by reduced density of Ca²⁺ release channels and/or an unexpected partial depletion of ER luminal Ca²⁺ stores. ALG-2 and Peflin expression levels decreased slightly, but the altered Ca²⁺ conditions caused no other expression changes in membrane trafficking machinery and had no detectable impact on ER stress. We conclude that in NRK cells at steady state, IP3Rs produce tonic Ca²⁺ signals that limit outer coat targeting to ERES, thus reducing the basal rate of ER export.

P932

A genetic screen in yeast identifies novel trafficking regulators of cell surface membrane proteins

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Many integral membrane proteins are transported to the cell surface where they perform diverse and critical functions. Various evolutionary conserved trafficking pathways transport surface protein cargoes to and from the plasma membrane, and therefore regulate surface protein activity. Perturbations in these pathways underlies many pathologies. We have developed a uracil-scavenging growth assay in yeast that reports on the surface activity of the plasma membrane uracil permease Fur4. This approach was validated using mutants that affect Fur4 trafficking, then used to perform a systematic genome-wide screen of viable haploid deletion strains. Of 5132 mutants tested, 150 mutants exhibited altered growth capacity under low uracil conditions. 126 mutants exhibited a growth defect in poor-uracil conditions, whilst 24 exhibited a growth advantage. Mutants involved in membrane trafficking and transcriptional regulation were particularly enriched. Future work includes microscopy-based approaches to test if the candidates with reduced growth capacity in poor-uracil conditions have defects in trafficking surface cargoes to the plasma membrane. Furthermore, we plan to test if the mutant candidates that exhibit enhanced growth in poor uracil conditions have elevated cargo export from the endoplasmic reticulum. In combination, we hope to validate all mutants that can be explained in the context of membrane trafficking and ascertain their mechanisms.
Spindle Assembly 2

P933

Post-transcriptional regulation during mitosis

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Translation is under tight spatial and temporal controls to ensure protein production at the right place and time. The project goal is to examine how post-transcriptional regulation mediated by microRNA-31 (miR-31) controls local translation to impact animal development. We have found that miR-31 is enriched in the perinuclear region of blastomeres in the interphase, and is associated with the mitotic spindles in blastomeres in mitosis. In order to understand the functions of miR-31 in development, we isolated miR-31 bound RNAs to identify potential regulated mRNAs and performed shotgun proteomics of control and miR-31 knockdown blastula stage sea urchin embryos to identify the proteins whose levels could be miR-31 regulated. Notably, these approaches have converged to indicate that the levels of several proteins that interact with actin, including Fascin, Rab35 and gelsolin are regulated by miR-31, while their corresponding mRNAs are also preferentially associated with the mitotic spindle during blastomeremitosis. This novel and striking oscillating localization of miR-31 and its targets suggests that these RNAs may play a direct role in spindle assembly, and/or cell cycle progression. The mitotic spindle is a highly dynamic structure consists of a large complex of microtubules and proteins that is regulated by actin and a complex set of proteins and RNAs. We found that miR-31 inhibition resulted in more extensive astral microtubules and less or incorrectly directed interpolar and kinetochore microtubules in cleavage stage embryos compared to the control. In addition, miR-31 inhibition led to increased Fascin protein perinuclearly in the non-dividing blastomeres and in the spindle midzone in dividing blastomeres, similar to miR-31 and Fascin transcript localization. These results indicate that miR-31 regulates local translation of actin remodeling genes on mitotic spindles to control microtubule dynamics and ensure proper early development.

P934

miR-31 regulates mitotic spindle formation by regulating actin dynamics

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miR-31 is a highly conserved microRNA that is known to play a role in cell proliferation, migration and differentiation. Using the sea urchin as a model organism, we observed that the mitotic spindle of miR-31 knock down (KD) embryos display longer and more disorganized astral microtubules. Using live-imaging techniques, we observed that miR-31 KD embryos also demonstrate defects in the orientation of the mitotic spindle. To understand the mechanism of these mitotic spindle defects, we identified several direct targets of miR-31 by injecting biotinylated miR-31 into sea urchin zygotes to pull-down its bound targets and using RNA-seq to identify differentially pulled down transcripts between control and injected embryos in the blastula. Additionally, the early blastula proteome from zygotes injected with a scrambled control or miR-31 inhibitor was submitted for mass spectrometry to identify miR-31 targets. Using dual-luciferase assays, we found that miR-31 suppresses several actin remodeling genes, including Rab35 and Fascin. Rab35 directs the polymerization of actin on the plasma membrane and Fascin bundles actin into linear arrays. Interestingly, miR-31, Rab35 and Fascin mRNA transcripts localize to the...
midzone of the mitotic spindle and to the perinuclear region in divided blastomeres. miR-31 KD results in an increase in Fascin protein at the mitotic spindle midzone and perinuclearly in divided blastomeres. These results suggest that miR-31 regulates local translation, particularly at the mitotic spindle. As previous research and our preliminary data have indicated that actin is important in mitotic spindle orientation and formation, we hypothesize that miR-31 regulates mitotic spindle formation through its direct regulation of genes that mediate actin. As the mitotic spindle is a highly dynamic structure that is responsible for ensuring daughter cells receive the correct complement of chromosomes during mitosis, defects in spindle orientation and formation may result in abnormal chromosome segregation, which often results in apoptosis, cancer or developmental defects.

P935

**Dissecting CG10126, a tubulin binding protein’s function in Drosophila cell.**

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Our lab is interested in epidermal growth factor receptor (EGFR)-directed cell proliferation in developing *Drosophila*. In a screen for transcriptional targets of EGFR activity, we identified a *Drosophila* gene, CG10126, that is upregulated 10-fold in response to EGFR activation. CG10126 is orthologous to human Calcyphosine (CAPS), whose expression is upregulated in many human cancer cells. Subsequent work from our lab reported that, CG10126 is a tubulin binding protein, promotes microtubule polymerization, localized along with mitotic spindle, and required to perform normal levels of mitosis. From these observations, we hypothesized that CG10126 might be required for regular cell cycle and aid in spindle stability to properly divide a cell into two new daughter cells. To test this hypothesis, we primarily, knocked down CG10126 using RNAi in cultured *Drosophila* S2 and GM2 cells, followed by immunocytochemistry and imaging for analysis. We also specify certain parameters to compare our control groups with the CG10126-dsRNA treated groups, which includes, cell viability, mitotic cell population, mitotic metaphase cell irregularity, cell population in between cell cycle and mitotic phases, spindle length and, regular bipolar and abnormal spindle phenotypes. During analysis, within our designated parameters, we observed significant difference in most of our experimental criteria. Our results suggest that, CG10126 is required for proper mitotic spindle machinery to ensure complete nuclear material separation in *Drosophila* cell and, hints about a similar role of CAPS in human cells. Above all, as there is high convergence between CG10126 and CAPS, these findings could be helpful to farther our understanding about fundamental aspects of mitosis, cell proliferation and clinical implications.

P936

**A novel mechanism that promotes mitotic spindle formation in human cells**

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The mitotic spindle consists mainly of microtubules (MTs) and is essential for accurate distribution of chromosomes to the two daughter cells during cell division. Errors in spindle formation can lead to incorrect separation of chromosomes or unequal size of daughter cells, which are associated with carcinogenesis or developmental disorders. However, the molecular mechanisms of spindle formation
are not fully understood. In the present study, we show for the first time that Chk1, a kinase involved in the cellular response to DNA damage, is essential for optimal density and effective polymerisation of the spindle MTs in human cells. Chk1 localises to the centrosomes (the main centers of MT organization) in mitosis and phosphorylates β-tubulin in newly identified sites in vitro. Also, reduced microtubule density in cells without functional Chk1 is associated with formation of disorganized spindles. We suggest that Chk1 phosphorylates β-tubulin to promote optimal spindle MT polymerisation. These findings describe a novel mechanism that could protect against carcinogenesis and developmental disorders, through regulating formation of the mitotic spindle.

P937

Ectopic Addition of Reticulon Impacts on Spindle Morphology
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Chromosome capture, alignment and segregation all depend on the mitotic spindle, one of the largest force generators inside cells. However, to which extend the spindle can be interpreted as mechanically isolated entity is still unclear. In small cells, spindle microtubules contact the cell boundary, which plays a role at least in spindle positioning. Other organelles and protein networks could also be involved in force balancing during chromosome segregation. In *Drosophila* syncytial embryos, the ER is excluded from the spindle, forming a seemingly elastic envelope. Although this membranous network surrounds the spindle throughout mitosis, its involvement in the mechanics of spindle assembly and chromosome segregation has been largely ignored. Conventional inactivation approaches are difficult to interpret owing to the importance of the ER for various cellular processes. To circumvent this experimental limitation, we fragment the ER in a specific and temporally controlled manner using microinjection techniques for *Drosophila* embryos. Reticulon-like protein 1 (Rtnl1) is an ER associated protein and determines membrane curvature and scission. Ectopic addition of Rtnl1 leads to ER fragmentation. Here, we show that acute addition of Rtnl1 during interphase leads accumulation the mitotic checkpoint protein - Mad2 - along microtubules and at spindle poles, and the cycle arrests at metaphase. Mad2 transport towards and accumulation at the poles suggests that the checkpoint silencing mechanism is active but the mechanical framework is altered, impairing release of Mad2 into the cytoplasm. To dissect the role of the ER on the maintenance of spindle integrity we acutely added Rtnl1 specifically in metaphase-arrested embryos. We found that the nearby environment is altered and that spindle length becomes progressively shorter. Moreover, the radial-like organization of the ER surrounding the centrosome is lost, suggesting that membrane disruption is more accentuated in this area. In parallel, we also found that Rtnl1 co-pelleted with Taxol-stabilized microtubules *in vitro*, suggesting a direct interaction of this protein with the spindle. Currently, we hypothesize that Rtnl1 could function as a microtubule-ER crosslinker. To test this, experiments are underway to determine whether the cytoplasmic domain of Rtnl1 is responsible for binding microtubules. We aim to understand whether Rtnl1 has a direct effect on spindle microtubules (altered microtubule dynamics) or whether the ER envelope itself can act as a structural scaffold for the mitotic spindle.
The communication between mitosis and cytoplasmic organelles in Caenorhabditis elegans
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The membranes of the endoplasmic reticulum (ER) surround and enclose the nucleus, forming a characteristic bi-membraned nuclear envelope that protects and structures the chromatin. Whether the ER also plays a role as a spatial confinement facilitating chromosome congressional and segregation during mitosis is not yet clear. To address this, we applied a genetic screen, light microscopy and large-scale electron tomography of Caenorhabditis elegans (C. elegans) one-cell stage embryo undergoing the transition from the prometaphase to metaphase stage. Here we report our characterization of a novel gene required for ER morphology and chromosome congression and alignment in C. elegans. During the first mitosis in mutant embryos the perturbation of ER membranes surrounding and enclosing the nucleus resulted in extensive clustering of ER, leading to abnormal chromosome congression/alignment. The mutant embryos had additional defect reminiscent of dynein/dynactin loos-of-function possibly caused by down-regulation of C. elegans dynein. To our knowledge, the novel gene is the first example of a link between ER and chromosome congression in C. elegans.

The role of the RhoGEF ARHGEF17 (TEM4) during mitosis
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Microtubules, which are dynamic filaments, have several roles in intracellular transport, cell motility and cell division. During mitosis, the assembly of a bipolar microtubule-based spindle, ensures accurate chromosome segregation, a crucial process required for genome integrity, due to well-controlled microtubule dynamics. The Dbl family of guanine nucleotide exchange factors (GEFs) are implicated in cytokinesis, the last stage of M phase (mitosis), but a recent study suggested that ARHGEF17 (TEM4), a Rho family GTPase exchange factor protein, is essential for the Spindle Assembly Checkpoint through targeting and acting as a timer for detention of Mps1 at kinetochores. TEM4 has been shown to contain a role in the interphase cytoskeleton, junction integrity and epithelial and endothelial cell function. In this study, we have confirmed the interaction between Mps1 and TEM4 and we are further exploring the role of TEM4 in mitosis. We detected TEM4 localizing at the mitotic spindle, the poles and the cytoplasm. TEM4 binding to actin in interphase is crucial for its subcellular localization and our work explores its additional role and potential binding to microtubules in mitosis. Indirect immunofluorescence confirmed that TEM4 localization is microtubule dependent and changes on the spindle microtubule dynamics, affect its localization. In addition, we have identified TEM4 as a new minus end binding protein, with uncharacterized functions so far. We are further exploring the effect of mitotic kinase on expression and localization of TEM4. Our preliminary results suggest that kinetochore proteins involved in microtubule binding, and kinases required for microtubule nucleation regulate TEM4 protein levels and localization. In this project, we seek to decipher the functions of a new minus end protein and determine the role of TEM4 in mitosis. This study will directly contribute to the
characterization of unknown mitotic functions of TEM4 related to the mitotic spindle and to a better understanding of how chromosome fidelity occurs. In addition, it will allow to explore novel approaches and therapeutic strategies against cancer.

P940

**Design and characterization of novel small molecule modulators of mitosis and spindle structure**

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Tight regulation of mitosis ensures genomic integrity and survival of daughter cells. The Spindle Assembly Checkpoint (SAC) is a regulatory mechanism that ensures that all chromosomes are bioriented before separation of sister chromatids begins. The cell will not proceed to anaphase until the SAC is satisfied by alignment of bioriented chromosomes on the metaphase plate. We have developed a novel class of potential HDAC inhibitors that have imidazole as the zinc-binding group. HDACs remove acetyl groups from histone and non-histone proteins to regulate multiple cellular processes including mitosis and the SAC. Because HDACs are mis-regulated in many types of cancer, neurodegenerative diseases, and some viral infections, they are attractive targets for developing new drugs. The compounds we developed inhibit purified HDAC isoforms in vitro with selectivity towards HDAC8. However, they do not affect histone acetylation when applied to cells. Surprisingly, they are toxic to various cancer cells in the low micromolar range. They induce mitotic arrest and disrupt spindle structure. Although they were designed as HDAC inhibitors the observed biological effects are likely due to alternative intracellular targets. Furthermore, cells are blocked in mitosis when exposed to the compounds at concentrations that have no effect on acetylation. HDAC8-null human cells are only partially protected from the compounds, indicating that HDAC8 is likely only one important target and there are additional targets contributing to the observed biological activity. Further analysis indicates that combining these compounds with Aurora B inhibitors causes catastrophic collapse of the mitotic spindle. Neither mitotic arrest, nor spindle collapse occurs with a panel of other HDAC inhibitors further suggesting that these mitotic effects are due to targets yet to be identified.

**Synaptic Functions**

P941

**The Spatial Scale of Synaptic Protein Allocation during Homeostatic Plasticity**

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An individual neuron hosts up to 10,000 individual synapses that can be made stronger or weaker by local and cell-wide plasticity mechanisms- both of which require protein synthesis. To address over what spatial scale a neuron allocates synaptic resources, we quantified the distribution of newly synthesized
proteins after global homeostatic upscaling using metabolic labeling and single-molecule localization (DNA-PAINT). Following upscaling, we observed a global increase in locally synthesized nascent protein in synapses and at dendrites, with a high degree of variability between individual synapses. We determined the smallest spatial scale over which nascent proteins were evenly distributed and found that it is best described by synaptic neighborhoods (~10 microns in length) - smaller than a dendritic branch and larger than an individual synapse. Protein allocation at the level of neighborhoods allows a neuron to balances local autonomy and global homeostasis.

P942

The ALFA-tag/Nanobody pair - a versatile tool for in vivo detection and functional reconstitution of synaptic proteins
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Synapses are communication units connecting cells in the nervous system and mediating efficient transmission of signals across the synaptic cleft. Our understanding of synapse architecture comes mostly from electron microscopy studies; the subsynaptic distribution of many components remains however obscure. Besides availability of suitable antibodies, the low level of synaptic proteins and the size and density of this microenvironment make localization studies very challenging. Fluorescent tags may interfere with the function of synaptic proteins. Furthermore, conventional immunohistochemistry using primary antibodies and amplification steps usually adds 20-30 nm to the distance between the epitope of interest and the detectable chromophore. With a length of 2-4 nm, nanobodies may offer a solution to this problem. Here we use a novel ALFA tag/nanobody (AT/NA) pair to examine the in vivo distribution and function of synaptic proteins at the Drosophila neuromuscular junction, a model system for glutamatergic synapses. We choose to examine Neurexin1 (Nrx1), a highly conserved cell-adhesion molecule critical for the assembly of presynaptic terminals. Nrx1 is a transmembrane protein with multiple extracellular modules, and a short intracellular domain bearing a C-terminal PDZ-binding motif. Guided by phylogenetic analysis and secondary structure prediction, we inserted the ALFA tag (AT) in between highly conserved intracellular motifs of Nrx1 and generated Nrx1-AT transgenes. Super-resolution microscopy revealed that neuronally overexpressed Nrx1-AT, visualized by ATTO-488 coupled nanobody ALFA (NA), co-localized perfectly with endogenous Nrx1 at the active zone. Furthermore, neuronal Nrx1-AT and Nrx1 but not Nrx1-GFP, rescued the lethality and physiological deficits of Nrx1null mutants, indicating that Nrx1-AT is fully functional. We edited the Nrx1 locus endogenously and generated a Nrx1-AT line which is indistinguishable from control and a Nrx1-ΔPDZ-AT allele (lacking the PDZ-binding motif) that resembles the Nrx1null. Nrx1-ΔPDZ-AT appeared restricted to the endoplasmic reticulum, indicating that the PDZ binding domain is required for surface delivery. Since the AT/NA pair binds with high affinity both inside and outside the cells in vitro, we tested whether a PDZ binding motif provided in trans could rescue the Nrx1-ΔPDZ-AT distribution and function. Indeed, presynaptic expression of NA-PDZ chimera restored the synaptic localization and rescued the physiological defects of Nrx1-ΔPDZ-AT. Thus, the AT/NA pair can be used for in vivo detection and functional reconstitution of a complex synaptic protein. The AT/NA pair emerges as a versatile in vivo tool for studying protein distribution and function within a tight and crowded cellular milieu.
**Divergence of the dendritic transcriptome in neurons**

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Neurons have highly polarized architectures which require complex molecular organization, partly mediated by RNA localization. Neurons leverage the localization of mRNA to modify synaptic features by locally translating and altering protein concentrations in response to stimuli. The resulting activity-dependent modifications are essential for synaptic plasticity, and consequently, fundamental for learning and memory. While a tremendous amount of work has been dedicated to identifying spatially controlled genes, only a few studies have examined the evolution of dendritic mRNA localization. Surprisingly, work by Francis et al. (2014) found that the dendritic transcriptome, the cohort of mRNA recruited into the dendrites, diverges greatly between mouse and rat, suggesting rapid evolution of a molecular process often considered to be highly conserved. Here, we used subcellular RNA-sequencing of dendrites and soma from the same Sprague-Dawley rat neurons to compare to a similarly detailed existing dataset for C57BL/6 mouse (Middleton et al. 2019). Analysis of the subcellular RNA-sequencing data identified 2,845 orthologous genes (1,537 and 2,093 in mouse and rat dendrites respectively) that are dendritically expressed in at least one of the two species. Interspecies comparisons of the dendritic transcriptomes revealed 821 orthologs with conserved localization, 716 orthologs being present in mouse dendrites only and 1272 orthologs in rat dendrites only, further characterizing the divergence in the subcellular localization of many genes. Subsequent analysis of the molecular evolution of genes in each localization category, along with 964 somatic genes for comparison, suggests higher rates of base pair substitutions in genes with divergent localization compared to those dendritically expressed in both species. In short, we not only confirmed Francis et al.’s observation that dendritic localization occurs in a species-specific manner but also identified sequence differences that might underlie the changes in subcellular expression of specific genes. Further analyses are currently underway to study the sequence evolution of RNA secondary structures that might signal for subcellular localization, as well as to find putative signaling subsequences that are evolving under directional or purifying selection. Regardless, the observed divergence of such an important neuronal process might hint at rapidly evolving, activity-dependent functional differences between neurons of closely related species.

**Deciphering the role of the formin mDia1 in oAβ1-42 synaptotoxicity**

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It is increasingly recognized that dynamic microtubules (MTs) play a crucial role for rapid forms of neuronal transport as well as in synaptic function. Whether defects in MT dynamics and organization at synapses play a pathogenic role in neurological disease remains however unknown. We recently found that activity-evoked de novo nucleation of dynamic MTs at excitatory presynaptic boutons is rate
limiting for synaptic vesicle (SV) exocytosis by limiting the intrabouton transport of SVs and SV precursors (Qu et al., 2019). In addition, previous work from our lab has underscored the importance of the formin mDia1 in mediating oligomeric amyloid beta 1-42 (oAb1-42) synaptotoxicity through the modulation of MT dynamics (Qu et al., 2017) and tau phosphorylation. Compelling evidence has implicated several formins in pre- and post-synaptic remodeling through regulation of actin and microtubule dynamics and a role in the SV cycle at presynaptic sites has been ascribed to mDia1 both in vitro and in vivo. The mechanism by which mDia1 acts as a permissive factor in oAb1-42 synaptotoxicity remains, however, unknown. We hypothesized that in pathological conditions, control of de novo MT nucleation at presynaptic sites may be hijacked by oAβ through mDia1 activation leading to synaptic injury and cognitive impairment. We examined the behavior of axonal dynamic MTs in cultured hippocampal neurons (18DIV) and found that induction of presynaptic MT nucleation and interbouton SV bidirectional transport was slowly triggered by oAβ1-42 in the absence of evoked neuronal activity and completely abolished by loss of tau expression or mDia1 inhibition, implicating this MT pathway in the induction of oAb1-42- and tau-mediated synaptic injury. To determine whether loss of mDia1 rescued the synaptic deficits caused by amyloid pathology in vivo, we crossed mDia1 KO mice with J20 mice and compared the four resulting littermate genotypes in hippocampal synaptic plasticity, spatial memory function and spine density. Strikingly, we found that even if mDia1 deficiency alone exhibited abnormal LTP, loss of mDia1 in J20/mDia1KO mice normalized LTP damage, memory deficits, spine density depletion and presynaptic synapsin 1 activation in J20 mice. Our findings reveal a novel role for mDia1 in mediating de novo presynaptic MT nucleation induced by oAβ1-42 and suggest that loss of its presynaptic MT activity in mDia1 KO mice may underlie the mechanism of synaptic rescue in mice affected by amyloid pathology.

Effects of early life adversity and monoaminergic systems on brain-wide coordination of neuronal activity
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Transitions of brain-wide activity patterns after an acute fear experience depend upon cellular interactions between monoaminergic systems and neurons of cortico-limbic systems. Drugs for mood disorders, such as anxiety and depression, target monoamine reuptake transporters located on pre-synaptic terminals of the highly evolutionarily conserved monoaminergic neurons. Early life adversity (ELA) may interact with development of these systems and thus underly the known vulnerability to mood disorders after ELA. To observe this complex dynamic, we couple transgenic mice, ELA, and predator stress (PS). We measured behavior in the light dark box, and brain-wide and cellular activity with manganese-enhanced magnetic resonance imaging (MEMRI) and optical microscopy. For ELA, dams were deprived of adequate bedding at P2-P9. Mice (10 weeks, n=24) with and without ELA were subjected to longitudinal MRI paired with behavioral recordings before, immediately and long after predator stress (Uselman et al., 2020). Defensive behavior was determined by time spent in the light. Mn2+, injected intraperitoneal (0.3 mmol/kg), enters neurons via voltage-gated Ca2+ channels and highlights active neurons by T1-weighted MRI. At the conclusion of imaging and behavioral recordings, mice were perfused with fixative, sacrificed and the brain removed, embedded, and serially sectioned.
for immunohistochemistry. MEMRI images were aligned and intensity scaled prior to statistical parametric mapping (SPM) and network connectivity analyses. Defensive behavior increased for both groups with PS (p<0.05, Tukey). Preliminary results show basal neural activity after ELA resembles that of acute fear after normal rearing. Although behavior in mice with and without ELA appear to recover 9 days after PS, brain activity remains altered. Neural activity detected by MEMRI was confirmed by staining for immediate-early gene, c-Fos. To test whether monoaminergic systems contribute to ELA phenotype, we performed parallel experiments in serotonin transporter knockout (SERT-KO) mice and MEMRI tract-tracing of projections from medial prefrontal cortex (mPFC) in WT, SERT-KO, norepinephrine transporter knockout (NET-KO), and ELA mice. Projections were differentially affected in SERT-KO, ELA, and NET-KO mice. To determine whether altered mPFC projections in ELA mice was due abnormal development of monoaminergic systems, we stained sections through the mPFC for serotonin and NET. ELA resulted in fewer processes and altered distribution of distal termini. Together our data finds that ELA disrupts arborization of noradrenergic projections in the mPFC and alters brain-wide coordination of basal neuronal activity and the response to fear both short and long-term. Support: RO1MH096093.

P946

Synaptic active zone assembly requires scaffold molecule phase separation

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In neuronal synapses, the active zone is the site of release of vesicles containing neurotransmitters. While the composition of active zones is well characterized, how active zone proteins assemble together and recruit synaptic release machinery is not clear. Here, we show that two widely conserved active zone components, C. elegans SYD-2/Liprin-α and ELKS, undergo a liquid-liquid phase separation that is critical to assemble the active zone. We first find developing active zones are dynamic and recover quickly after photobleaching, consistent with a possible phase separation. In contrast, mature active zones become highly static with nearly zero cytoplasmic exchange. We find that SYD-2/Liprin-α and ELKS, two core active zone scaffolding proteins, are capable of liquid-liquid phase separation in vitro and in vivo, and rely upon multiple disordered motifs for this activity. By selectively mutating these motifs, we are able to specifically ablate phase separation activity without affecting the scaffolding functions of the proteins. We endogenously introduced the SYD-2 and ELKS phase separation-blocking mutations in vivo with CRISPR/Cas9, and find they localize normally, but lack fluid dynamics even in developing synapses. Consequently, we find additional active zone components fail to assemble properly and synaptic vesicles fail to cluster, causing synaptic transmission and behavioral defects. Critically, when SYD-2’s phase motif is replaced with that of an unrelated phase separating protein, FUS, assembly of the active zone is rescued, confirming the central importance of phase separation activity. Finally, we reconstituted SYD-2 and ELKS phase-separated scaffolds in vitro, and find they are able to incorporate additional active zone components UNC-10/RIM and GIT. We find this incorporation depends on SYD-2 and ELKS fluid dynamics; SYD-2 and ELKS-1 condensates that have matured and solidified, or condensates of mutants with decreased dynamics, are unable to evenly incorporate UNC-10 and GIT. Our data show that presynaptic active zones are assembled through a phase separation of core scaffold molecules. This phase separation is critical for the accumulation and incorporation of active zone components at a nascent synapse before maturation into a stable structure.
**X-linked recessive mutations in PDZD4 are likely associated with neurodevelopment delay and central nervous system abnormalities.**

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Neurodevelopmental disorders (NDD), a group of diseases that affect the development of the central nervous system, can occur from a multitude of genetic predispositions and environmental factors. Despite the high frequency of NDDs in children, little is understood about the mechanism by which they lead to disease. Genetic abnormalities are considered the strongest risk factors for NDD and have been demonstrated to affect multiple facets of brain development and function. Molecular genetics is attempting to determine the roles of various genes that may be involved in NDD. In this study, we describe a pediatric patient who presented with learning disability, autistic features, and developmental delay. Exome sequencing re‐analysis identified a hemizygous truncating nonsense mutation in PDZD4, which we showed segregated with disease by Sanger sequencing. We also confirmed decreased mRNA expression of PDZD4 gene in patient fibroblasts compared to controls. While we know that PDZD4 protein and mRNA are expressed highly in brain tissue, little is known about PDZD4 function, except that it shares a common PDZ domain with other protein families that are abundant and essential in signal transduction systems. PDZ-domain-containing-proteins have been linked to processes such as ion-channel signaling, transport, and neuronal development. Essential information on how these domains influence synaptic messaging, however, remains unknown. For these reasons, we chose an in vitro human model to investigate the phenotype of the neural network and examine communication among neurons. We used CRISPR to investigate the functions of PDZD4, knocking down genes in i3N, an induced pluripotent cell (iPSC) line that has an inducible Neurogenin-2 expression, allowing for scalable production of glutamatergic neurons. Using this cell model, we successfully knocked down the transcription of PDZD4 and performed RNaseq on the iPSC-derived neurons. RNA seq analysis when compared to control showcased over 600 differentially expressed genes. Using pathway analyses, we observed that many of these genes are involved in central nervous system processes, including synaptogenesis and axonal guidance signaling, highlighting the importance of PDZD4 in nervous system development. We plan to further analyze the morphological differences of this patient and others with similar X-linked mutations in PDZD4 using our established cell model. Our work will be useful in elucidating the role of the PDZD4 gene and may provide a better understanding of NDD.

**Reward-specific ensembles in the Nucleus Accumbens core**

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Poorly regulated reward seeking is a central feature of substance use disorder. Convergent findings using different biomarkers reveal that only ~2-5% of cells encode a putative cocaine ensemble. When animals are exposed to two rewards, *in vivo* measurements of neuronal firing in the nucleus accumbens reveal ~20% overlap between neurons responding to self-administration of different types of reward such as cocaine, water, regular chow or sucrose, while a study using FISH reports that 50% of activated neurons in the infralimbic prefrontal cortex respond to both ethanol and saccharin. These results suggest a finely tuned specificity of ensembles. Here we comprehensively characterize the specific ensembles of neurons built through experience that are linked to two antagonistic behavioral strategies: seeking and extinction learning. We additionally address the question of whether or not addictive drugs usurp the neuronal networks recruited by natural rewards by evaluating cocaine- and sucrose-associated ensembles within the same animal. We use targeted recombination in active populations (TRAP) strategy, specifically FosCreERT2+/Ai14 (cFos-TRAP) transgenic mice to deposit a cFos-driven Cre recombinase-tdTomato reporter into neurons activated during cue-induced reward seeking and extinction in order to tag cells as potentially encoding these behaviors. To characterize the seeking and extinction ensembles, these mice underwent the well-described rodent behavioral model of cocaine self-administration (SA), extinction training and cue-induced reinstatement of seeking. To define and compare different reward-specific ensembles within the same animal, we developed a poly-reward cocaine and sucrose self-administration paradigm in mice, where each reward is associated to a different discrete cue. After undergoing extinction training in absence of cues, mice are first re-exposed to one cue in presence of 4-OHT, allowing the tagging of the reward-specific ensemble with tdTomato, and a few days later exposed to the second cue, followed by immediate Fos tagging. Using this paradigm, we were able to assess the neurons included in the cocaine or sucrose ensembles, and to quantify the overlap between the two populations within the same animal exposed to both types of reward. We tagged ~1% of neurons in the core subregion of the accumbens activated during cue-induced seeking for cocaine or sucrose. The majority of tagged cells in the cocaine- or sucrose-seeking ensembles were D1-MSNs, and specifically activated during seeking. Using the cocaine and sucrose poly-reward model, we found ~70% distinction between the cells constituting the cocaine- compared to the sucrose-seeking ensemble.

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**Semaphorin3A and PlexinA3 association with a scaffold for cGMP increase is required for apical dendrite development**

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The development of the apical dendrite from the leading process of the bipolar pyramidal neuron might be directed by spatially organized extrinsic cues acting on localized intrinsic determinants. The extracellular cues that regulate apical dendrite polarization have remained elusive. We find that leading process and apical dendrite development is directed by class III Semaphorin signaling and mediated by a localized cGMP-synthesizing complex. We show that the scaffolding protein Scribble, which associates with cGMP-synthesizing enzyme soluble-Guanylate-Cyclase (sGC), also associates with the PlexinA3 coreceptor for Semaphorin3A (Sema3A). Deletion or knockdown of PlexinA3, Sema3A, or disruption of
PlexinA3 - Scribble complex associations prevented Sema3A-mediated cGMP increase and caused severe defects in apical dendrite development in the embryonic CA1. These manipulations also caused defects in bipolar polarity and leading process establishment. Together, we show that during neuronal polarization, leading process formation and subsequent apical dendrite development is directed by a scaffold that links extracellular Semaphorin cue to cGMP increase.

P950

Tiam1 is critical for glutamatergic synapse structure and function in the hippocampus

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Mounting evidence suggests numerous glutamatergic synapse subtypes exist in the brain, and that these subtypes are defined by unique molecular regulatory mechanisms. Studies of molecules that regulate glutamatergic synapses are typically confined to Schaffer collateral-CA1 pyramidal neuron synapses, whereas little is known about the molecular mechanisms that confer unique properties to perforant path-DG synapses. We find that the expression of the Rho guanine-nucleotide exchange factor (GEF) Tiam1 is restricted to the dentate gyrus and that it plays a unique role in the regulation of synaptic development at perforant path-DG granule neuron synapses but not Schaffer collateral-CA1 pyramidal neuron synapses. Our data shows that inhibition of Tiam1 function in dentate granule neurons reduces synaptic AMPA receptor function and causes dendritic spines to adopt a filopodia-like morphology. We find that Tiam1’s support of perforant path-DG synapse function is dependent on its GEF domain and identify a role for Tiam1’s auto-inhibitory PH domain in regulating Tiam1 function at these synapses. Together, these data identify a critical role for Tiam1 in the hippocampus and reveal a unique Tiam1-mediated molecular program of glutamatergic synapse regulation in dentate granule neurons.

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Pro-bdnf changes in the fronto-cerebellar circuitry after oxycodone-induced place preference in adult rats

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Introduction: Recent studies support the involvement of the cerebellum in addiction. Opioid receptors mu- and kappa-varieties are present in the cerebellum; nonetheless, the effects of oxycodone in the cerebellum are unknown. Brain-Derived Neurotrophic Factor (BDNF), the precursor proBDNF, and the matureBDNF regulate opioid induced-plasticity in the mature brain; thus, it may be pivotal in oxycodone rewarding properties and responses. Aim: Determine the effects of oxycodone induced-conditioned place preference (CPP) on levels of Pro-BDBF/mature-BDNF in the prefrontal cortex and cerebellum. On PND120, male adult rats were tested for CPP after being randomly assigned to the oxycodone drug group (four daily 20-min pairings with oxycodone (3 mg/kg, s.c.) and four daily pairings with saline on alternate days) or the vehicle group (eight 20-min pairings with saline). Following conditioning, rats were given a 20-min CPP test for evaluating oxycodone preference. After the preference test, brains were collected and analyzed for Pro and Mature BDNF levels with an immunoassay (ELISA). Results: Oxycodone induced CPP [ F (1, 21) = 55.02, p < .001]. Furthermore oxycodone induced-CPP increased levels of Pro-BDNF in the PFC [ F (1, 21) = 14.46, p < .001] and decreased levels in the cerebellum [ F (1,
Conclusions: Oxycodone CPP modified Pro-BDNF levels in a “seesaw” mechanism in the fronto-cerebellar circuitry. Thus, pro-BDNF may be required neurotrophins in the opioid-induced reorganization of fronto-cerebellar circuitry and fronto-cerebellar dysfunction.

The Nuclear Envelope and Disease

P952

Nuclear pore complex quality control in neurodegeneration

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A G4C2 hexanucleotide repeat expansion (HRE) in the C9orf72 gene is causative of ALS and FTD. Recently, we have demonstrated that pathologic repeat RNA species arising from this HRE leads to a reduction in the nuclear levels of 8 specific components (nucleoporins, Nups) of the nuclear pore complex (NPC) in C9orf72 induced pluripotent stem cell (iPSC) derived spinal neurons (iPSNs) and postmortem patient tissue. Here, we have uncovered the underlying mechanisms that lead to this Nup loss and disruption of NPCs in neurodegeneration. We provide evidence that CHMP7 and VPS4, components of an ESCRT pathway linked to NPC quality control, are dramatically increased in C9orf72 iPSN nuclei prior to the emergence of Nup alterations. Consistent with a role in regulating Nup levels within NPCs, knockdown of CHMP7 mitigates disease-associated injury and restores the nuclear levels of specific Nups in human neurons. Additionally, by specifically inhibiting the nuclear export of CHMP7, we can trigger Nup reduction. Thus, our data support a role for disruptions in CHMP7 and ESCRT-III mediated Nup homeostasis in mammalian nuclei and C9orf72 mediated disease pathogenesis.

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Identification and characterization of transketolase as an autoantigen within the dense fine speckled 70 (DFS70) defined nuclear region


Background: Anti-nuclear antibodies (ANA) against intracellular antigens is associated with systemic autoimmune rheumatic diseases (SARD). Using HEp-2 cell based indirect immunofluorescence assay (HEp-2 IFA), the International Consensus on ANA Patterns (ICAP: www.ANApatterns.org) defined 30 anti-cell (AC) patterns. AC-2 defined as nuclear dense fine speckled pattern is associated with antibodies to DFS70, a 70kDa protein which is a coactivator of nuclear transcription. As the majority of individuals with anti-DFS70 antibodies are healthy subjects, anti-DFS70 antibodies can serve as a biomarker to rule out diagnosis of SARD. Objective: The aim of this study was to investigate related autoantibodies that produce AC-2 pattern independent of anti-DFS70. Methods: Human sera tested by Inova HEp-2 ANA slides showing AC-2 pattern were graded 1-4 based on staining intensity. These samples were then retested using ImmoHEp-2 ELITE kit with DFS70 knockout cells (HEp-2/DFS70 KO IFA) according to the manufacturer’s instructions. Three representative sera with characteristic AC-2 pattern using both HEp-2 slides and negative for anti-DFS70 by enzyme-linked immunosorbent assay (ELISA) were selected for immunoprecipitation (IP). Validation experiments were performed using available monoclonal antibody and recombinant protein in Western Blot and ELISA. Results: IP performed using HeLa lysate and mass
spectrometry analysis revealed that Transketolase (TKT) was common to all three representative AC-2 positive and anti-DFS70 ELISA negative sera analyzed. To validate that anti-TKT antibodies are distinct from anti-DFS70, monoclonal anti-TKT antibody and TKT recombinant protein were obtained for further analysis. IP-Western Blot using monoclonal anti-TKT antibody confirmed anti-TKT was present in all three sera. Monoclonal anti-TKT antibody produced an AC-2 like pattern in interphase HEp-2 cells, but only showed heterogeneous staining of the metaphase plate in mitotic cells. By HEp-2/DFS70 KO IFA, anti-TKT antibody produced an AC-2 like pattern in interphase cells and mitotic cells were strongly stained. Double staining with patient serum and monoclonal anti-TKT antibody produced the same staining pattern as when antibodies were stained separately. **Conclusion:** TKT is validated as an autoantigen co-localizing with DFS70 in interphase nuclear dense fine speckles and partially on metaphase cells. TKT association with metaphase plate is enriched in DFS70-depleted cells. Further experiments are needed to establish the exact role of TKT in DFS70 associated function.

**Mechanisms of skeletal muscle and and cardiac disease caused by mutations in TMEM43**

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Dominant mutations in the human TMEM43 gene cause Emery-Dreifuss muscular dystrophy (EDMD)-related myopathy, which is characterized by muscle weakness and wasting and joint contractures. Dominant mutations in TMEM43 also cause arrhythmogenic right ventricular cardiomyopathy (ARVC), characterized by adipose tissue accumulation and fibrosis, primarily in the right ventricle, leading to arrhythmias and sudden cardiac death. TMEM43 encodes the integral inner nuclear membrane protein TMEM43, also called LUMA. It is unclear if mutations in TMEM43 cause similar cellular dysfunction in skeletal muscle and cardiac tissue. To better understand the the function of TMEM43 in muscle, we used the Drosophila Gal4/UAS system and RNAi knock-down. An RNAi transgene against CG8111, the Drosophila orthologue of TMEM43 (designated dTMEM43), was expressed either in the larval body wall muscle, adult indirect flight muscle, or cardiac tissue. Knock-down of TMEM43 in larval body wall muscle reduced viability, with death occurring at the pupal stage. Larval body wall muscles exhibited chromatin protrusions at myotendinous junctions where physical forces are the greatest, suggesting a weakened nuclear envelope. Knock-down of TMEM43 in indirect flight muscles caused wing posturing defects characteristic of loss of indirect flight muscle function. Knock-down of TMEM43 in cardiac tissue resulted in defective wing heart function, leading to blistered wings, and premature death in adulthood. Viable adults showed an age-dependent elevated heart rate, a shortened diastolic interval, and an accumulation of adipose tissue around the heart compared to controls, similar to the human disease phenotypes. Taken together, these data demonstrate that many of the human muscle disease phenotypes caused by mutation in TMEM43 are recapitulated in Drosophila, providing a new model to identify mechanisms of disease. These studies are supported by the Undiagnosed Disease Network.
Nesprin-1 recruitment of microtubule cytoskeleton components to the nuclear envelope underlies the pathology in striated muscle laminopathies

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Mutations in the nuclear envelope (NE) protein lamin A/C, encoded by LMNA, result in muscular dystrophy and dilated cardiomyopathy (DCM). SYNE1, encoding the KASH domain NE protein Nesprin-1, has also been reported to be mutated in DCM. KASH domain proteins physically interact with SUN domain proteins to form the NE-spanning LINC (Linker of Nucleoskeleton and Cytoskeleton) complexes. To investigate KASH-dependent Nesprin-1 function, we used CRISPR/Cas9 to mutate the NE-anchoring KASH domain in mice, thereby disrupting Nesprin-1-containing LINC complexes. In cells, derived from homozygous Syne1Kfs (KASH frameshift) mice, Nesprin-1 is mislocalized from the NE. In the Syne1Kfs multi-nucleated myotubes and cardiomyocytes, the nuclei were mispositioned, with centrosome, kinesin, and dynein components being mislocalized from their usual location at the NE. However, no overt physiological defects were observed in these mice. To investigate potential genetic interactions between Lmna and Syne1, Lmna mutant mice, that had cardiac or striated muscle defects, were crossed with Syne1Kfs mice. Double mutant mice survived 2 to 5 times longer than Lmna mice. Disruption of Nesprin-1-containing LINC complexes therefore ameliorates the pathological and cytotoxic effects of Lmna mutations. This suggests LINC complex disruption is a therapeutic target for the laminopathies. Given the mislocalization of microtubule components from the Syne1Kfs NE, we propose that microtubule-based forces transmitted by the LINC complex impinge on stress-sensitized Lmna mutant nuclei to cause disease.

Age-associated Nuclear Remodeling Contributes To Cardiac Dysfunction

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Cardiac performance depends on the proper function of the extracellular matrix, cytoskeleton and nucleus to regulate systolic contraction and diastolic relaxation. As we age, the mechanical properties of these structures change, yet their influence on tissue-level function is unclear. Nuclear Lamins (Lam), intermediate filaments underlying the nuclear envelope, change in expression and localization with age and, when mutated, cause cardiomyopathy. We hypothesized that age-associated LamA/C reduction remodels nuclear shape, force propagation, and gene accessibility to reduce cardiac function. We profiled cardiomyocyte nuclei from rapidly aging w1118 and yw wildtype Drosophila melanogaster strains, and found that they became smaller and more circular with age, in contrast to skeletal muscle nuclei. Expression of both LamB and LamC (Drosophila LamA/C) decreased with age and differentially regulated nucleus size and circularity. However, LamC knockdown, but not LamB, induced contractile dysfunction, measured by fractional shortening, at 1-week, and shortened lifespan. Lamin knockdown mimicked DNA
decondensation observed during aging, suggesting that reduced DNA packaging could result from fewer contacts with the nuclear lamina. We subsequently have developed a novel ATAC-seq pipeline, optimized for frozen heart nuclei that will identify how age-related nuclear architecture may influence the epigenome and contribute to pathogenic heart remodeling. Together, we show cardiomyocyte-specific nuclear remodeling occurs as a result of diminishing Lamin expression and induces age-related cardiac dysfunction. In future work, we aim to elucidate the mechanism by which loss of nuclear Lamin contributes to age-related cardiac dysfunction.

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A biophysical approach to investigate cell mechanics in premature aging disease
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Lamin A/C is an intermediate filament included into a meshwork lining the inner membrane of the nucleus. While ensuring the nucleus mechanical stability and regulating chromatin organization and transcription factors, lamin A/C plays a key role in mechanotransduction. Mutations in the lamin A/C gene (LMNA) have been related to a variety of human diseases with varying degree of severity, collectively called laminopathies. Laminopathies share common symptoms such as predisposition to cardiovascular disease, fat storing issues and premature aging. In the most severe case, Progeria, all cell types are affected and the entire body ages prematurely. But the full relationship, from the molecular level to the entire body, between LMNA mutations, mechanotransduction and disease severity remains unknown, resulting in a lack of diagnosis and treatment. We chose to investigate at the cell level the link between LMNA mutations and alteration of viscoelastic properties, such as cortex tension, cytoplasm viscosity or nucleus rigidity. Previous studies aiming to evaluate cellular viscoelastic properties either lack quantification or statistics due to a low number of cells. To bridge this gap, we developed a microfluidic device in which cells are transiently stressed in narrow constrictions. We optimized a drug treatment on healthy cells to mimic premature aging phenotypes observed in cells from patients carrying LMNA R482W mutation. This allows us, firstly to compare a model of premature aging to laminopathic cells and, secondly to correlate phenotype with accelerated aging. We assessed the response to mechanical constraints of healthy and aging cells, showing that the latter are more viscous than the control cells. Finally, disruption of the cytoskeleton components showed that the actin network affects more the cell response than microtubules. Our results are currently discussed in the frame of a physical model which considers the respective contributions of cytoplasm and nucleus. Our study highlights that both nucleus and cytoplasm control the passage through narrow constrictions, and that premature aging related to LMNA mutations increases the effective viscosity of the cell.
Therapeutic Targeting of Cancer Stem cells

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Retinoids to target cell differentiation in colorectal cancer
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Colorectal cancer (CRC) arises through acquisition of mutations in a colonic stem cell (SC) that transforms it into a cancer stem cell (CSC), which drives tumor development. There are no curative treatments for advanced CRC because CSCs are resistant to chemotherapy and/or radiation. Because all-trans retinoic acid (ATRA) is a highly effective and often curative treatment for acute promyelocytic leukemia patients, we conjecture that retinoids may be an efficacious treatment for CRC. Retinoic acid (RA) is a metabolite of vitamin A which is known to induce differentiation of SCs and inhibit cell proliferation. Our goal is to discover how to therapeutically target human CRC CSCs using the differentiation-inducing effects of retinoid agents. Our previous study on the RA metabolizing enzyme aldehyde dehydrogenase (ALDH) shows that ALDH marks normal and malignant colonic SCs and that RA signaling mainly occurs through colonic ALDH+ SCs. Hypothesis: The ability of various retinoid agents (ATRA, 9-cis retinoic acid and Liarozole) to induce differentiation of CSCs depends on the RA pathway genotype according to mutations in RA signaling genes in CRC cells. Indeed, preliminary data from our bioinformatics analysis shows that many RA pathway genes are overexpressed and often mutated in CRC. We are now determining the effects of RA ligands on cell growth and differentiation using a panel of CRC cell lines that have specific RA signaling mutations. Further investigation of RA signaling mechanisms that regulate colon SCs and how dysregulation contributes to the SC overpopulation that drives CRC growth should provide insight into strategies for designing new SC-targeted therapies for CRC.

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Cell cycle genes mediating FOLFOX resistance in FOLFOX resistant CD133+ colorectal cancer cells
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Colorectal cancer (CRC) in South Africa, is the third most common cancer in women and the second most common in men. 5-Fluorouracil (5-FU) and oxaliplatin are established chemotherapies used in the treatment of metastatic colorectal cancer. However, due to tumor recurrence, both drugs fail to improve the 5-year survival rate after treatment. Cancer stem cells (CSCs) are a distinct subset of cells residing within a tumor, which contribute to the initiation, maintenance, and therapeutic resistance of cancer, leading to patient relapse. Initially characterized as slow-cycling, long-lived cells that reside in discrete histological niches, CSCs are highly proliferative non-niche multilineage progenitors of several cell types. To understand and characterize the differential and drug selective expression of CRC CSCs, we evaluated the cell cycle transcriptome of the 5-FU and oxaliplatin resistant CRC CSCs, compared to their parental CSCs. A concentration series of 5-FU and oxaliplatin was used to gradually induce in vitro drug resistance in HT29 colorectal adenocarcinoma cells. Three separate HT29 populations were drawn as 5-FU resistant (5-Flu HT29, resistant to 20µM 5-Flu), Oxaliplatin resistant (Oxa HT29, resistant to 20µM
Oxa) and as drug sensitive parental class (Parental, IC50 of 5-Flu=1µM and Oxa=1 µM). Each population was exposed to the alternate drug and cell cycle response was measured using PI staining in a Muse™ Cell analyser. CSCs isolated from each population using CD133 magnetic beads were grown and exposed to 5-Flu and Oxa; and the real time quantification of cell cycle transcriptome was performed using RT² Cell Cycle Array Profiler. Significantly altered gene expression was further confirmed using SYBR-Green reporter assays in qRT-PCR reactions; and transcriptome analysis was performed using a ∆∆Ct approach. The most differentially key expressed cell cycle genes identified, RBL2, BCCIP and SKP2 were studied using reverse genetics in vitro. Both gene knock down and knock out strategies were employed to evaluate gene function(s) by analysing the FOLOX susceptibility in HT29 cells. siRNAs directed against RBL2, BCCIP and SKP2 were used in a series and combinations of transfection in CRC CSCs; and FOLFOX susceptibility was established using Alamar blue staining. CRISPR/Cas9 gene editing was used to knock out mainly the promoter area and the area of functional binding site in the ORFs of the three key genes. In a series of different combinations of CRISPR/Cas9 plasmid transfections, CSCs showed significant alternations on the susceptibility of CSCs to the FOLFOX drugs.

P960

Anticancer Activities of Spiro(N/O) and 2-cis-4-Ansa(N/O) Cyclotetraphosphazene Derivatives

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The octachlorocyclotetraphosphazene, N₄P₄Cl₈, is the well-known starting compound for the preparation of cyclotetraphosphazene derivatives in the phosphazene chemistry. Reactions of N₄P₄Cl₈ with bidentate reagents such as dialkoxides and diamines are rarely encountered in the literature [1]. In the last two decades, aziridine, pyrrolidine, piperidine spermine, spermidine and 4-fluorobenzyl diamine-substituted cyclotriphosphazenes have been found to be potential anti-cancer agents. Some of the aminophosphazenes have cytotoxic activities against to mouse P388, L1210, B16, HT-29, Vero, Hep2 and HeLa cells [2]. However, there are few studies on the antimicrobial and anticancer activity studies of cyclotetraphosphazenes [3]. In this study, the substitution reaction of N₄P₄Cl₈ with an equimolar amount of sodium 3-(N-ferrocenylmethylamino)-1-propanoxide yielded two types of products, namely, mono-ferrocenyl-spiro- (spiro) and mono-ferrocenyl-2-cis-4-dichloro-ansa-(2,4-ansa) cyclotetraphosphazenes. The reaction of these products with the sodium salt of excess N-(4-fluorobenzy1)-N’-ethylene-1,2-diamine and sodium 3-(4-fluorobenzylamino)-1-propanoxide resulted in the formations of spiro(N/O) and 2-cis-4-ansa(N/O) cyclotetraphosphazenes with bulky (4-fluorobenzyl) N/N and N/O donor type bidentate ligands. Furthermore, this study mainly focused on in vitro cytotoxic activity studies of the novel cyclotetraphosphazene derivatives against L929 mouse fibroblast, Caco-2 colorectal adenocarcinoma and A549 non-small lung cancer cell lines. According to the cytotoxic activity results, it was determined that seven compounds showed similar cytotoxic activity to
Inhibition of NADPH oxidases impairs the characteristics of cancer stem cells

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Cancer stem cells (CSCs) exhibit self-renewal properties, tumorigenicity, and multilineage differentiation capacity and have been found to be the sole reason for cancer relapse. The NADPH oxidases (NOXs) are a family of multicomponent enzymes that are key producers of reactive oxygen species (ROS). The precise role of NOX enzymes in cancer stem cell remains poorly defined. But there are evidence that the NADPH oxidase support the features of CSCs such as tumorigenesis, metastasis, and metabolism. Recently our group converted iPSCs into CSCs using conditioned media (CM) that mimic tumor inducing microenvironments which serves as a model of carcinogenesis. In this study, we investigate the effect of NOX inhibitor, Diphenyleneiodonium (DPI), on CSC deriving from iPSC cells by assessing cell proliferation, self-renewal, migration and stemness in vitro. The first line of our results indicates that IC50 of DPI for iPSC derived CSCs is much higher in comparison to cell lines in the previous reports. The inhibition of NOXs showed significant reduction of self-renewal and stemness and alters the expression of CSC markers. The results indicate that NOX is essential for maintaining the specific characters of CSC. Therefore, targeting NOXs might be an effective method to target CSCs for future therapeutics. Simultaneously, it will be a clue to understand the significance of ROS as the second messenger of CSC maintenance.

Modulation of growth and stemness in colorectal cancer cells by tetrazole dithiocarbamate derivatives

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Resistance to therapies occurs in almost all patients with colorectal cancer (CRC), particularly in those with metastatic tumours. Associated with drug resistance and tumour recurrence are cancer stem cells, a tumour subset population. To combat recurrent CRC, novel therapeutic strategies involving the discovery of novel anticancer drugs targeting cancer stem cells are required. We therefore evaluated anticancer activity of a novel series of ten synthetic tetrazole dithiocarbamate derivatives (TDTCs) in CRC cells and explored their modulatory anticancer effects on CRC stem cell biomarkers. Anticancer activity of ten novel TDTCs were assessed by an initial qualitative screening using Alamar Blue, followed by a quantitative cell death counting using Trypan blue; and the inhibitory concentrations (IC50) were determined in HT29 and DLD1 CRC cells. CRC cells were monitored for their growth response to tested TDTCs using the Muse cell cycle assay and the xCelligence real time cell analyser. The CRC stem cells
were assessed for their ability to form spheroids in response to the TDTCs using real time microscopy imaging. The effect of the active TDTCs on CRC stem cell markers was evaluated using a proteome profiler assay and real time PCR. Four TDTC’s designated 1, 2, 4 and 5 were found to be the most active compounds (IC50<0.1 to 0.4mg/ml against CRC cells. Theses TDTCs reduced cell proliferation as shown by real-time cell impedance analysis. Cell death was induced ranging from a level 66%-68% in CRC cells, while in non-cancerous HEK293 cells, only 9%-2% cell death was observed. Cell cycle arrest was promoted at different phases in HT29 and DLD1 cells, affecting G0/G1 and S-phase, dependent on the TDTC used. With regards to stemness, the ability of HT29 CD133+ cancer stem cells to form spheroids was either reduced or impeded by TDTC treatment. Protein profiling disclosed increased expression of E-cadherin in HT29 and DLD1 CD133+ stem cells; while FoxA2 was down regulated and Sox17 was upregulated in the HT29 CRC stem cells. Gene expression analysis revealed downregulated β-catenin, VEGF1, VEGFR1 and FoxA2 expression in CRC stem cells. Little or no expression of these markers was found in the CD133 negative CRC cells. In conclusion, active TDTCs were potent anticancer agents affecting cell growth and modulating biomarkers in CRC stem cells.

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Novel insights into the relationship between cellular prion protein and glioblastoma stem cells maintenance and resistance to temozolomide

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Glioblastoma multiforme (GBM) is one of the most lethal cancer types, being significantly invasive and aggressive. This glioma has several malignant features and an impressive cellular heterogeneity, presenting a subpopulation of stem-like cells - denominated glioblastoma stem cells (GSCs) - which have been related to GBM maintenance, aggressiveness and resistance to therapy. GSCs are resistant to the most common anti-GBM chemotherapeutic drug, the alkylating agent temozolomide (TMZ), through a mechanism that requires the action of methylguanine-DNA methyltransferase (MGMT), a protein involved in DNA repair that is upregulated by hypoxia-inducible factor 1α (HIF1α). Our group has studied the roles of cellular prion protein (PrP<sup>C</sup>) and its partners in the biology of GSCs, and we have proposed PrP<sup>C</sup> as a scaffold protein able to integrate a signaling platform on the plasma membrane involved in stemness. PrP<sup>C</sup>, encoded by PRNP, is upregulated in GBM and is associated with its aggressive behavior, being involved in proliferation, self-renewal and tumorigenicity of GSCs. Remarkably, recent evidences point out to a crosstalk between PrP<sup>C</sup> and HIF1α. Given the importance of PrP<sup>C</sup> in the biology of GSCs and its relation with this hypoxia-related protein, we have analyzed public bulk transcriptome data from The Cancer Genome Atlas (TCGA) in order to elucidate whether prion protein might be involved in GSCs maintenance and in GBM resistance to TMZ via HIF1α. After normalizing gene expression counts, we divided TCGA GBM samples into two groups with distinct PRNP expressions (PRNP-Low and PRNP-High) and assessed whether this variation affected the expression of genes of interest and patient survival. Strikingly, MGMT gene expression is augmented in PRNP-High relative to the PRNP-Low group, suggesting a correlation between PRNP expression and TMZ resistance. The gene that encodes DOT1L, a relevant protein in GSCs biology, has a decreased expression in the PRNP-High group, and SOCS3 gene, also involved in GSCs maintenance, shows no statistically significant alteration between the groups. The Nestin-encoding gene, NES, does not display a statistically significant variation either. These results are consistent with preliminary data from our group. Additionally, survival analyses demonstrate that patients from PRNP-High group had a poorer survival than those from PRNP-Low, and that a high
expression of PRNP combined with high expressions of HIF1α and MGMT has a notable negative impact on survival. Together, these results provide new insights into the roles of PrP\(^{C}\) in GBM stemness and resistance to therapy, and highlight important intracellular signaling pathways for future experimental analyses.

P964

**Establishing a link between WNT and retinoic acid signaling in colorectal cancer development**

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The focus of our lab has been to understand how *adenomatous polyposis coli* (*APC*)- mutation leads to decreased stem cell (SC) maturation and colonic SC overpopulation in the development of colorectal cancer (CRC). Based on our studies, we hypothesize that human colorectal cancer (CRC) evolves due to an imbalance between WNT and retinoic acid (RA) signaling. Indeed, we observed that sequential inactivation of *APC* in familial adenomatous polyposis (FAP) patient tissues leads to progressively delayed neuroendocrine cell (NEC) maturation of aldehyde dehydrogenase- positive (ALDH\(^+\)) SCs during CRC development. Given that ALDH is a key component of the RA signaling pathway and since *APC* mutation causes increased WNT signaling in FAP, we decided to investigate a possible link between the two pathways. Accordingly, we conducted in vitro experiments using human CRC cell lines to determine whether *wild-type* (*wt*)-*APC* can restore the ability of *APC*-mutant CRC cells to undergo NEC maturation, reduced cell proliferation, increased sensitivity to retinoids, and reduced Wnt/\(\beta\)-catenin signaling. Upon inducing *wt*-APC, we found an increase in the protein expression of several NEC markers including CHGA, GLP2R, NSE (ENO2), and SSTR1; concentration-dependent increased sensitivity of CRC cells to ATRA-induced inhibition of cell proliferation; and decreased WNT/\(\beta\)-catenin signaling via TCF reporter assay. In addition, *wt*-APC restored degradation of \(\beta\)-catenin and decreased protein expression of several TCF4- targets known to be upregulated by WNT signaling activity: MYC, JUN, MET, and CD44. Thus, our findings indicate that both WNT and RA signaling pathways are affected by mutated APC and may explain how APC mutation leads to decreased maturation of SCs and the cancer SC overpopulation that drives CRC development and growth.

**Wednesday, December 16, 2020, 11:00 am**

**Actin and Actin-Binding Proteins in Mechanobiology and Engineering 2**

P965

**\(\alpha\)-Actinin and Fascin Sort to Assemble Distinct Actin Network Architectures in Confined Space**

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Actin binding proteins (ABPs) are assembled in specific domains of the actin cytoskeleton to control actin network organization and remodeling throughout the cell. Among ABPs, actin crosslinkers determine the local geometry and architecture of actin networks which enable actin-based mechanical function of the cell. Actin crosslinkers, α-actinin and fascin, are highly enriched in, respectively, contractile actin networks and cell surface to regulate cell mechanosensation and motility. By encapsulation of actin with α-actinin and fascin in giant unilamellar vesicles (GUVs), we show that actin filaments form distinct structures. While actin bundles merge into a single actin ring in small GUVs, they form aster- and star-like structures in large GUVs. Depending on the degree of confinement, α-actinin-mediated aggregation and bundling determine actin architecture. However, competition between α-actinin and fascin for crosslinking, which results in their sorting and domain formation, contributes to the centering of actin aggregates and formation of rigid star-like structures. By measuring aggregate size and flexural rigidity of actin bundles at different concentrations of crosslinkers, we show that α-actinin-rich actin aggregates are segregated from fascin domains which form rigid star-like bundles radiating from the aggregates. A simulation framework demonstrates how crosslinker size and density is sufficient to drive the formation of star-like structures for energy optimization in a confined space. Our results contribute to a better understanding of mechanical basis for self-organization in cellular networks as well as the design of cytoskeleton-based minimal cells.

Regulation of actin turnover by myosin activity and network architecture
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Actin remodeling through turnover is essential for the survival of most cells. However, the regulation of this process through actin architecture and myosin activity is poorly studied. While myosin-mediated disassembly of actin filaments has been implicated in regulating turnover in cells (Wilson et al. Nature 2010), the underlying dynamics of this process, and the effects of actin structure, have not been studied. Here, we investigate the ability of myosin activity and actin architecture to control turnover. Using a minimal system to recreate turnover in vitro, we observe that severing by coflin increases the turnover rate in our networks. We additionally find that myosin mediated severing is sufficient to increase the rate of actin turnover, even in systems without coflin. Preventing filament depolymerization through the addition of phalloidin or reducing myosin-mediated buckling by shortening the length of filaments reduces this effect, suggesting that the increased rate relies on actin severing and disassembly. This increase in turnover, however, is dependent on actin structure. When actin is bunded by the crosslinker α-actinin, cofillin mediated turnover vanishes. Remarkably, α-actinin does not impact myosin mediated severing, and myosin is able to increase turnover even in bundled networks. These results suggest that not only can myosin regulate turnover of actin filaments, but also that different methods of disassembly might be needed to remodel actin depending on its local structure.

Functional optimality of the highly conserved Arp2/3-mediated 70° branching angle
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Actin-based motility is widespread among eukaryotic cells. Many distinct cell types must be able to undergo migration reliably in order to perform a variety of biological functions. However, the fundamental mechanism by which actin produces force, the Brownian ratchet, carries with it an inherent stochasticity. We aimed to understand how robust migration behavior can arise from stochastic actin polymerization in the context of a particularly resilient type of protrusion, the lamellipodium. In previous experimental and theoretical efforts, we developed a stochastic model of the leading edge that faithfully recapitulates the stable lamellipodial shape dynamics observed in migrating cells. The stability of this minimal model, in the absence of any additional feedback mechanisms, necessarily implies that lamellipodial maintenance is an intrinsic property of branched actin growth against a membrane. In the present work, we performed a theoretical exploration into the biophysical mechanisms underlying stability in our model. Surprisingly, we find that membrane tension is dispensable for maintaining a stable leading edge, and instead identify lateral filament spreading, mediated by the inherent geometry of branched actin growth, as the critical contributor to lamellipodial stability. Furthermore, we find that the genetically encoded branch geometry, the Arp2/3-mediated 70-80° branching angle that has been conserved from protists to mammals, maximally suppresses leading edge shape and actin density fluctuations. Taken together, these results contextualize two long-standing experimental observations: First, they suggest that the essential lamellipodial maintenance role of the Arp2/3 complex is rooted in its ability to facilitate filament spreading and suppress stochastic fluctuations. Second, the results hint that the high conservation of the Arp2/3-mediated branching angle may stem from its capacity to optimally perform this smoothing effect. In conclusion, we find that the branched structure of actin at the leading edge naturally quells stochastic fluctuations, thereby representing a novel biological noise-suppression mechanism based entirely on system geometry.

P968

Crowding tunes the organization and mechanics of actin bundles induced by actin crosslinking proteins


Actin crosslinking proteins such as fascin and α-actinin form higher-ordered actin bundles that mediate numerous cellular processes including cell morphogenesis, adhesion, and movement. While it is understood crosslinked bundle formation occurs in crowded cytoplasm, how crowding affects the bundling activities of the two crosslinking proteins is not known. We hypothesize that changes in filament mechanics and conformations in the presence of crowding may affect fascin and/or α-actinin binding to filaments, thereby modulating subsequent bundling activities. Utilizing total internal reflection fluorescence (TIRF) microscopy and atomic force microscopy (AFM) imaging, we demonstrate that macromolecular crowding affects the packing of both fascin- and α-actinin-bundles and has a stronger impact on fascin bundle stiffness than on α-actinin bundle mechanics. Fascin induces stiffer bundles than α-actinin, and the bending stiffness of fascin-induced bundles exhibits sensitive dependence on the types as well as concentrations of crowders. All-atom molecular dynamics (MD) simulation results support the inference that crowding reduces the binding interactions between actin filaments and fascin or the calponin homology (CH) 1 domain of α-actinin evidenced by interaction energy and hydrogen bonding analysis. Based on our findings, we suggest a mechanism of crosslinked actin bundle assembly and mechanics in crowded intracellular environments.
Counteractive Effects of Electrostatics and Macromolecular Crowding on Actin Bundle Mechanics and Secondary Structure

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Actin bundles are a critical component of the eukaryotic cytoskeleton which contributes to the structural support of the plasma membrane and cell motility. Bundle formation occurs in crowded intracellular environments containing high concentrations of ions and many macromolecules. While the roles of electrostatic interactions or macromolecular crowding in actin bundling have been independently well established, how they act together to compact and stabilize or induce structural transitioning of bundles thereby altering mechanics has yet to be understood. In this study, we use a combination of total internal reflection fluorescence (TIRF) microscopy and Fourier transform infrared (FTIR) spectroscopy to investigate the effects of various ionic and crowded conditions on the stiffness and secondary structure of bundles. Utilizing TIRF microscopy, we demonstrate divalent cations and macromolecular crowders result in enhanced bundle stiffness, reduced bundle lengths, and alterations to bundle thickness. Furthermore, FTIR allows us to evaluate the secondary structure and stability of actin bundles induced by cations or macromolecular crowders over a wide range of crowding or ionic concentrations. Our spectroscopy analysis indicates bundles undergo shifts in bond vibration, signifying distinct molecular interaction forces stabilizing bundle structure. Taken together, our work suggests electrostatic and entropic forces may work counteractively and cause transitioning of secondary structure and bending stiffness of actin bundles.

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Turnover, mechanics, and structural plasticity of lamellipodial actin networks

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The dendritic network of actin filaments that provides the force for lamellipodial protrusions has been a powerhouse system for quantitative cell biology. We developed filament-level kinetic and mechanical models to study key questions in lamellipodial dendritic actin structure, turnover, and mechanics that remain poorly understood. Electron microscopy studies revealed a dense brushwork composed of short filaments near the leading edge followed by longer and more linear filaments near the center and rear. Single molecule imaging experiments have indicated frequent actin assembly and disassembly throughout the lamellipodium. Our three-dimensional kinetic model includes mechanisms for polymerization, depolymerization, branching, capping, uncapping, severing, oligomer diffusion, annealing, and debranching. We show that frequent severing and annealing can account for the structural change in the actin network, as well as the measured single molecule lifetimes. To understand how force transmits through the actin network, we studied a 3D Brownian dynamics model with retrograde flow maintained by pushing forces from the leading edge due to actin polymerization, pulling forces due to molecular motors, and frictional forces at nascent focal adhesions. We simulate the tension and bending distributions of actin filaments and the change from compressive stress close to the leading edge versus extensile stress at the back. Filament bending close to the severing threshold (as revealed in prior experiments) is seen around simulated nascent focal adhesions. Network connectivity
requires a sufficient number of permanent and temporary crosslinkers, which can also bias the formation of filopodial- or arc-like bundles.

P971

**Modeling cells as pressurized elastic shell**

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Animal cells and bacteria are enveloped and sealed by lipid membranes and mechanically protected by cortical polymer networks. Cells typically actively maintain a small (eukaryotic cells) or large (prokaryotic cells) positive osmotic pressure against their environment. Volume and shape regulation impact the mechanical properties of cells. The mechanical properties of cells can be probed by exerting external force and measuring cell response. To interpret micro-mechanical optical trapping experiments with suspended rounded eukaryotic cells, we developed finite element simulations and modeled cells as pressurized elastic shells. During deformation, competition between osmotic pressure resulting from compression of the cytosol and the elastic stretching of the actin cortex determines the cell response. The finite element simulations suggest that (eukaryotic) cell deformations are essentially isovolumetric.

**Cadherins and Cell Mechanics**

P972

**Function of ectoderm-mesendoderm interaction during lateral mesendoderm migration in early zebrafish gastrula**

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At the onset of gastrulation, mesendoderm progenitors undergo cell internalization at the germ ring margin and migrate collectively between the yolk membrane and the ectoderm layer towards the animal pole of gastrula to then converge towards the forming head-tail (body) axis. Fate mapping experiments showed that the future head-tail axis of the fish is roughly laid down along the dorsoventral axis of the germ ring, with the most anterior part of the body axis corresponding to the dorsal germ ring margin. Concurrently, mutants affecting migration during gastrulation display defects either in cell fate specification or in the correct ratio of each fate. To understand the interplay between cell fate specification and migration, and whether biophysical forces play a role therein, we are studying lateral mesendoderm migration during early gastrulation. Our preliminary results show that, regardless of their future cell fate, (i) lateral mesendoderm cells can undergo directed migration towards the animal pole and (ii) use the overlying ectoderm, which moves in the opposite direction, as the substrate for the migration. We are now analyzing the nature of the interaction between lateral mesendoderm and ectoderm and whether it plays a role in the initial phase of migration.
Medio-apical tensions regulate tight junctions of well-polarized Madin-Darby Canine Kidney cells

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Tight junctions, which locate at the apicolateral borders of epithelial cells to seal the intercellular space, are previously proposed to connect to the circumferential actomyosin belt, whose contractility exerting line tensions affects the paracellular transport by opening pores of tight junctions. Although tight junctions are known to display tortuous morphology, the line tension leads straight tight-junction morphology as energy minimization. To resolve the conflict, here we hypothesized that tight junctions are connected to the apical actin cortex, whose contractility exerts an in-plane surface contraction to the tight junctions. The tensions with an orthogonal component to tight junctions can explain both tortuous morphology and pore opening. To test this hypothesis, we laser ablated a spot in the apical surface of a well-polarized Madin-Darby Canine Kidney cell. The ablation destructs the contractile apical actin cortex and thus reduces the in-plane tensions, which leads to an decrease in the apical actin associated proteins, an increase in the apical areas and a decrease in the tortuosity of tight junctions. We observed the changes of MDCK cell sheets due to cell proliferation and ROCK inhibitor Y27632 perturbation and confirmed the trends of increasing apical actin cortex and decreasing tight junction tortuosity. Surprisingly, our morphological analysis further revealed scutoids on flat cell sheets, which were unexpected based on predictions from a previous model that only considered the cell-cell interactions as line tensions. The additional cell-cell interaction from apical in-plane tension could explain the existence of scutoids on flat geometry.

Investigating Vinculin’s role in mediating tension transmission at tricellular junctions in vertebrate epithelia

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Epithelial tissues are critical for generating compartmentalized barriers that separate internal and external environments to ensure proper organ function. Apical cell-cell junctions, including adherens junctions (AJs), which promote cell-cell adhesion, and tight junctions (TJs), which allow for selective permeability, are essential for epithelial structure and function. Recent research has revealed that mechanosensitive proteins like Vinculin are recruited to AJs to reinforce the connection between AJs and actomyosin when tension is applied on epithelia. However, little is known about how epithelial tissues maintain junctional integrity and barrier function when mechanical force is locally applied on cell-cell junctions. To investigate Vinculin’s role in mediating tension transmission at sites of locally-increased tension, we are using the Xenopus laevis embryonic epithelium, which exhibits naturally-occurring local increases in tension - at the cleavage furrow during cell division and at tricellular junctions (TCJs) where three cells come together - and can be manipulated to generate global increases in tension. We found that at baseline tension, Vinculin is localized in three “spots” surrounding the TCJ, and when tension is increased, Vinculin recruitment is increased, forming three elongated “spokes” around the TCJ. Using FRAP experiments to investigate the stability of Vinculin at these sites, our preliminary results indicate that at baseline levels of tension, Vinculin is stabilized at TCJs when compared to bicellular junctions (BCJs). In current work, we are investigating the effects on Vinculin...
stability at TCJs and BCJs when tension is globally increased by treating the embryo with extracellular ATP or Calyculin A. Furthermore, we are testing the effects of perturbing Vinculin function. When a dominant negative mutant of Vinculin (Vinculin-D1) is overexpressed, TJ proteins are reduced specifically at TCJs, indicating that Vinculin is needed to stabilize these structures. In work in progress, we are knocking down Vinculin and investigating changes in junctional protein localization and dynamics, actomyosin organization, and barrier function at TCJs at both baseline and globally-increased tension. Together, these experiments will provide novel insights into Vinculin’s role in maintaining cell-cell adhesion at AJs under increased local tension and will provide valuable information about the interplay between cell-cell adhesion and barrier function.

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Structural Dynamics of Homophilic Binding of Cadherin Anchored to Supported Lipid Bilayer Revealed by High-Speed Atomic Force Microscopy

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Homophilic binding of epithelial classical cadherin (E-cadherin) through their ectodomains is essential to epithelial cell-cell adhesion. The adhesive functions of E-cadherins are conserved among vertebrate and invertebrate animals, while the domain organization of the ectodomains is highly diversified. The ectodomains of vertebrate E-cadherin are composed of five consecutive extracellular cadherin (EC) domains, referred to as EC1 to EC5, and form a slightly curved, rod-like structure. The membrane-distal EC1 of vertebrate E-cadherin acts as an interface of trans-homophilic binding between cells. An epithelial classical cadherin in the insect *Drosophila melanogaster* is *Drosophila* E-cadherin (DE-cadherin), whose ectodomains are composed of seven consecutive EC domains and following non-EC domains. DE-cadherin is the functional counterpart of vertebrate E-cadherin, but the structural mechanism for its homophilic binding is unclear. Using high-speed atomic force microscopy (HS-AFM) in solution, we previously showed that the membrane-distal four EC domains (EC1-EC4) of DE-cadherin form a knot-like structure and are essential for mediating homophilic binding specificity by cell aggregation assay (Nishiguchi et al., 2016). However, the HS-AFM observation failed to visualize binding pairs of purified DE-cadherin ectodomains probably because the membrane-proximal side of them were not anchored to the substrate. In this study, we developed a reconstitution method to observe binding pairs and their dynamic behavior using supported lipid bilayer (SLB) and identify homophilic binding of DE-cadherin by HS-AFM. We sparsely deposited SLB on the mica substrate and anchored the membrane-proximal side of DE-cadherin to it. This method enables us to visualize detailed structure and dynamics (nano-meter and sub-second resolution) of cadherins anchored to SLB on an interface between SLB and mica surface. We directly visualized transitions between monomer and dimer structure of DE-cadherin, and found that the knot-like portion of DE-cadherin serves as the interface of homophilic binding, as previously hypothesized. The presented method will facilitate investigations of dynamic mechanisms for homophilic binding of cadherins by HS-AFM.
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*Rap1 controls adhesion and cytoskeletal rearrangements to drive rapid wound repair*

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Collective cell movements contribute to tissue development and repair, as well as the spread of disease. We investigate collective cell migration during wound healing in the *Drosophila* embryonic epidermis. Embryos repair wounds rapidly, through the coordinated movement of the cells around the wound. Upon wounding, the cells immediately adjacent to the wound become polarized: cell-cell adhesion molecules are internalized from the wound edge, and actin and the molecular motor non-muscle myosin II accumulate at the interface with the wounded cells, forming a supracellular cable around the wound that coordinates cell movements. The cable is thought to assemble from and anchor at the former tricellular junctions (TCJs) along the wound edge, which are reinforced by cable contraction. However, the mechanisms that regulate the molecular rearrangements that drive wound closure are unclear. The small GTPase Rap1 is a mechanosensitive molecular switch that promotes cytoskeletal polarization, interacts with actin regulators, and regulates cell-cell adhesion turnover. Therefore, Rap1 may coordinate the adhesion and cytoskeletal rearrangements that drive wound repair. To test this, I used genetic manipulations to modulate Rap1 activity. Reducing Rap1 activity by overexpressing a dominant-negative Rap1 slowed wound repair by 68%. The decrease in wound closure rate was accompanied by a 69% decrease in myosin accumulation at the wound edge. Conversely, increasing Rap1 activity by overexpressing a constitutively-active Rap1 resulted in wounds that repaired 14% faster and accumulated myosin 3-fold faster than in controls. Our results indicate that Rap1 controls myosin dynamics during embryonic wound healing. In addition, we found that Rap1 and E-cadherin were simultaneously depleted from the wound edge and localized together to TCJs during wound closure. Reducing Rap1 activity did not affect removal of E-cadherin from the wound edge but led to a 35% reduction in E-cadherin accumulation at TCJs, indicating a role for Rap1 in directing E-cadherin localization to TCJs. Thus, our data support a model in which Rap1 is responsible for the feedback between actomyosin cable assembly and reinforced adhesion at TCJs - both of which are necessary to drive rapid wound repair.

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*Mesoscale cadherin puncta are interdigitated protrusive actin microspikes*

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Cadherins build stable, cohesive sheets of cells using paradoxically weak bonds. Actin is thought to convert weak binding into strong adhesion either by exerting myosin dependent pulling forces on adhesive junctions or by clustering cadherins in the plane of the membrane. In contrast, actin polymerization dependent pushing forces are thought to serve only as a repair mechanism to fix broken junctions. Here, however, we show that actin polymerization dependent protrusions serve as the primary mechanism through which actin stabilizes cell-cell adhesion. Lateral membranes of epithelial
cells are continuously pushed against each another in the form of small protrusions. Cadherin puncta, long thought to be clusters of cadherins, turn out to be patches of protruding microspikes that form interlocking fingers of cadherins holding neighboring cells together. When polymerization is blocked, protrusions cease, puncta disappear, and lateral membranes detach from one another. In contrast, inhibiting myosin II contractility has no effect on adhesion.

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Emerged Oscillation by Nutrient-Modulating Growth Feedback
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Growth feedback, the inherent coupling between the synthetic gene circuit and the host cell growth, could significantly change the circuit behaviors. Previously, a diverse array of emerged behaviors, such as growth bistability, enhanced ultrasensitivity, and topology-dependent memory loss, were reported to be induced by growth feedback. However, the influence of the growth feedback on the circuit functions remains underexplored. Here, we reported an unexpected oscillatory behavior of a self-activation gene circuit induced by nutrient modulating growth feedback. Specifically, after diluted the activated self-activation switch into the fresh medium with moderate nutrient, its gene expression first decreases as the cell grows and then shows a significant overshoot before it reaches the steady states, leading to oscillation dynamics. The mechanism of the oscillation was demonstrated by a new mathematical model, which includes the ribosome allocation towards gene production, cell growth, and cell maintenance. Interestingly, our model predicted a counterintuitive dependence of oscillation amplitude on the nutrition level, where the highest peak was found in the medium with a moderate nutrient but not a rich nutrient. We experimentally validated this prediction by tuning the nutrient level with various fractions of LB in the culture medium. Our results demonstrated a new nonlinear emergent behavior mediated by growth feedback, which depends on the ribosome allocation between gene circuit and cell growth.

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Modeling asynchronous nuclear division in Ashbya Gossypii
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Multinucleate cells are common in biology, with examples including muscle cells, placenta, and fungi. Despite this, many aspects of their cell biology are not well understood. Dividing nuclei residing in a common cytosol would be expected to synchronize, as the oscillating levels of cell cycle regulators from each nucleus should in theory entrain neighbors. However, in the multinucleate fungus Ashbya Gossypii, spatially neighboring nuclei have been observed to divide out of sync. Here we mathematically model Ashbya nuclei as a dynamically growing system of coupled phase oscillators to determine possible mechanisms that could lead to asynchronous division. Nuclear movement in space is modeled to
capture core features of Ashbya nuclear dynamics, including both repulsion of and rearrangement with neighbors. We study the effects of mobility, cytosolic compartmentalization, inhibitory signals, and noise on transient phase dynamics. To compare the model with experimental results, we develop a nuclear tracking pipeline with the aim of tracking nuclei during bypassing events, identifying nuclear division, and linking nuclei into hyphae. Initial modeling results suggest a combination of locally and globally acting mechanisms might be at play leading to the observed asynchrony in Ashbya.

P981

**In Silico Predictions of Nucleolin-miRNA interactions via Molecular Modeling and Docking**

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RNA binding proteins (RBP) oversee and regulate many important cellular processes through their interactions with RNA molecules. Dysregulation of RBPs and RNA metabolic pathways is an established hallmark of tumorigenesis. Nucleolin (NCL) is a stress responsive multifunctional RBP with established roles in ribosome biogenesis, chromatin remodeling, microRNA(miRNA) processing, and transcriptional as well as post-transcriptional gene regulation. These functions of protein NCL are achieved through RNA-interactions driven by its four RNA binding domains (RBDs). Studies from literature suggest a growing list of miRNA molecules that interact with NCL in many human cancers, yet mechanisms that drive NCL-miRNA interactions remain unknown. This gap in knowledge is further compounded by partial three-dimensional structural information available for NCL RBDs and most miRNAs. In this study, we analyzed NCL-miRNA interactions in depth with a focus on a subset of miRNAs that are implicated in breast cancer. We have built upon the existing NCL-RBDs structures to generate multiple combinations of RBDs in tandem, and three dimensional models of the selected miRNA using in silico modeling approaches. Models with top validation scores were used in RNA-protein docking algorithms and assessed for interaction with specific miRNA. We then created a comprehensive map of the miRNA-NCL interface for each individual miRNA, based on docking analyses. Our results consistently predict RBD3-4 as the key drivers of NCL-miRNA interaction. We have also identified 3 distinct miRNA - NCL RBD interaction modes encompassing aromatic and positively charged residues in RBD 3-4. Our results corroborate previous studies that suggest the requirement of ribonucleoprotein (RNPs) motifs in RNA-binding and extends to identify unique residues on NCL RBDs. The study outcome thus enables us for the first time to establish consensus motifs on NCL-RBDs that can drive miRNA target specificity. The in silico predictions from this study will help design rational experiments for future studies to validate the proposed mechanisms of NCL-miRNA interactions and lay the foundations towards exploring NCL-miRNA interactions as potential drug targets that could be utilized in development of cancer therapies.

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**Computational modeling of chromosomal instability and analysis of phylogenetic topology enables inference of chromosome missegregation rates**

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Chromosomal instability (CIN), the persistent asymmetrical segregation, or missegregation, of chromosomes, is a hallmark of human cancer and major driver of intratumoral heterogeneity (ITH).
Moderate levels of CIN are associated with advanced clinical features of cancer and worse patient prognosis. Despite the clear clinical significance of a tumor’s baseline rate of chromosome missegregation, we still have no clinically accessible quantitative measure of this process. This knowledge gap is further highlighted by the possibility of baseline CIN as a biomarker for chemotherapeutics that increase CIN such as the spindle-poison, paclitaxel. This is largely due to the reduction in apparent karyotypic heterogeneity in a tumor via karyotypic selection. To this end, we applied agent-based computational modeling of chromosome missegregation rates and selective pressures on a population scale. We found that merging two models of aneuploid cellular fitness to account for both the negative selection experienced by aneuploid cells and the positive selection of pro-tumorigenic karyotypes allowed us to recapitulate the karyotype-level clonality often seen in tumors via single cell sequencing. Measurement of both the phylogenetic topologies and karyotypic diversity of hundreds of thousands of these simulated tumors evolved under a broad distribution of chromosome missegregation rates provided a dataset against which we can compare experimentally and clinically derived single cell DNA sequencing. In this way, chromosome missegregation rates can be inferred in these samples. We tested this experimentally in a high-CIN, low-selection context by treating CAL-51 cells with 20 nM paclitaxel and performing single cell copy number sequencing (scCNseq) after 48 hours. By manually quantifying the incidence of non-modal chromosomes we calculated a per division chromosome missegregation rate of 18±7 (0.41±0.16 chromosomes). Quantifying karyotypic diversity and features of phylogenetic topology in this same data and comparing it against our simulations with approximate Bayesian computation set gave a chromosome missegregation rate of 17.4 (0.40). We conclude that this method can accurately quantify CIN in low-selection conditions and may be extensible to clinically derived scCNseq.

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Mapping cell structure across scales by fusing protein images and interactions
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The eukaryotic cell is a multi-scale structure with modular organization across at least four orders of magnitude. Two central approaches for mapping this structure - protein fluorescent imaging and protein biophysical association - each generate extensive datasets but of distinct qualities and resolutions that are typically treated separately. Here, we integrate immunofluorescent images in the Human Protein Atlas with ongoing affinity purification experiments from the BioPlex resource to create a unified hierarchical map of eukaryotic cell architecture. Integration involves configuring each approach to produce a general measure of protein distance, then calibrating the two measures using machine learning. The evolving map, called the Multi-Scale Integrated Cell (MuSIC 1.0), currently resolves 69 subcellular systems of which approximately half are undocumented. Based on these findings we perform 134 additional affinity purifications, validating close subunit associations for the majority of systems. The map elucidates roles for poorly characterized proteins, such as the appearance of FAM120C in chromatin; identifies new protein assemblies in ribosomal biogenesis, RNA splicing, nuclear speckles, and ion transport; and reveals crosstalk between cytoplasmic and mitochondrial ribosomal proteins. By integration across scales, MuSIC substantially increases the mapping resolution obtained.
from imaging while giving protein interactions a spatial dimension, paving the way to incorporate many
molecular data types in proteome-wide maps of cells.

P984

**Effect of Cargo-Motor Dissociation on transport properties of molecular motor ensemble: A Semi-
Analytical Approach**

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Intracellular transportation involves multiple molecular proteins working together in coordination.
These proteins are of multiple types, i.e. they have different mechanochemical cycles. Understanding
transport properties of these molecules individually as well as of their ensembles help understand the
underlying mechanism of genesis of neurodegenerative diseases arising from defects in motor
transport. There has been an increasing interest in understanding how different types of motors
coordinate and how a multiple motor ensemble behaves as well as determining key factors impacting
the cargo transport phenomenon inside the cell. In the past, Monte-Carlo as well as Markov Chain based
semi-analytical approaches have been used to simulate the coordinated movement by ensembles of
molecular motors. Semi-analytical approaches have been shown to be less computationally intensive
while being capable of providing insights about rare-event scenarios. However, both these approaches
have assumed a fixed number of motors on the cargo. Recent experimental studies show that
attachment/detachment rates of the motor on/from the cargo play an important role in the cargo
transport by single motor or multiple motor ensemble. To investigate and quantify the effect of cargo-
motor dissociation on motor transport, we develop a Markov-chain based simulation strategy, which
assumes the number of motors on the cargo to be a stochastic quantity. Motors attach and detach from
the cargo with certain rates in addition to stepping, attaching, and detaching on/from the cytoskeletal
tracks. These rates, along with the maximal number of motors possible on a cargo, are varied, and their
effect is observed on transport quantities like average run lengths, average velocities, and average
number of motors engaged in carrying the cargo. The results are compared with, and studied against the
studies which assume a fixed number of motors on the cargo and are used to obtain better insights
about experimental findings. Preliminary results show that probability of dissociation has a significant
impact on run lengths with a twofold decrease in average run lengths for a 5-fold increase in probability
of dissociation of motor from the cargo.

Cytokinesis: Unicellular

P985

**Contractile ring tension and septum shape mechanically regulate each other in fission yeast**

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A long term goal is for cytokinesis research is to develop fully integrated models that explain contractile
ring constriction and accompanying processes in integrated mechanistic pictures. Contractile rings divide
cells, but regardless of the organism ring constriction is coupled to other systems (e.g. extended actin
cortex, plasma membrane or cell wall). Fission yeast ring constriction is tightly coupled to synthesis of
new cell wall as an ingrowing septum in the wake of the constricting ring. We built such a quantitative 3D computational model for fission yeast, integrating ring constriction and septum shape evolution, which revealed a fundamental force-mediated interdependence: ring tension is a 3D septum growth coordinator, while septum shape regulates ring organization. The contractile ring model is molecularly explicit with known amounts, organization and interactions of key components. The septum component is a 3D stochastic growth model evolving septum shape and growing the septum inwards. The integrated description incorporates mechanosensitivity of plasma membrane-bound septum-growing glucan synthases (Thiyagarajan et al., 2015). The model revealed mechanoregulation in both directions. The ring regulated septum shape via mechanosensitivity of the glucan synthases, suppressing septum shape irregularities and maintaining a smooth nearly circular ingrowing septum edge. A different mechanosensitivity, due to ring tension pulling cell wall growers to the septum tip, regulated the entire septum to be coplanar by suppressing out-of-plan undulations and ensuring opposite inner septum edges eventually met each other for proper septum closure. Thus, contractile ring tension serves as a 3D septum growth coordinator. Regulation also occurred in the opposite direction. The septum edge controlled the ring by setting ring shape. Provided the septum was regularly shaped, this maintained the ring organization and tensile functionality. In simulations of myo2-E1 mutant cells, reduced tension perturbed the ring organization through positive feedback: irregular septum edges destabilized ring organization, with thick actomyosin bridges peeling off in a runaway instability that fed back destructively into septum shape. This interdependence has been experimentally demonstrated: myo2-E1 cells have irregular septa and inhomogeneous glucan synthase distribution (Zhou et al., 2015). Synthase mutations also produces misshapen septa and rings with segments separated from the septum (Munoz et al., 2013). Finally, fitting simulated septum cross-sectional shapes to electron microscopy images (Munoz et al., 2013) revealed that primary septum growth occurs in a ~ 150 nm tip region and is ~ 50-fold faster than secondary septum growth on the septum sides. 1

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Cofilin protects contractile rings from bridging instabilities
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We show that a major role of the severing agent ADF/cofilin is to protect contractile rings from unanchoring bridging instabilities, the principal instability threatening the structural integrity and tensile functionality of rings. ADF/cofilin achieves this by limiting actin filament length and hence filament tension, which limits the forces tending to pull filaments away from their myosin II anchors. Significant experimental evidence from fission yeast studies suggests the major structural challenge faced by the cytokinetic contractile ring is bridging instability. When uncontrolled, this instability causes sections of the actomyosin ring to pull away from the membrane into straight bridges, suggesting the major task of ring-membrane anchors is to combat such instabilities. Such straight bridges were observed in rings with the myosin-II mutant myo2-E1, which binds actin weakly (Laplante et al., 2015). Bridges have also been observed in rings with the ADF/cofilin mutants adf1-M2 and adf1-M3 (Cheffings et al., 2019), which impair cofilin-mediated severing of filamentous actin. Sections of the ring pealed away from the plasma membrane into straight bridges with actin and myosin II Myp2, leaving myosin II Myo2 behind on the plasma membrane. More bridging occurred in regions of lower Myo2 density. To examine the origin of bridging, and the role of ADF/cofilin in stabilizing the ring, we used molecularly explicit simulations of the fission yeast ring constrained by measured amounts of key components and with Myo2, formin
Cdc12, and others organized into membrane-anchored nodes. In simulations, since actin filaments under tension have a strong energetic preference to be straight, the ~ 400-800 pN ring tensions generated high centripetal (“Laplace”) forces tending to unanchor the ring from the membrane. These were opposed primarily by binding of membrane-anchored Myo2 to the tense actin filaments, drastically weakened in the myo2-E1 mutant. Simulations of myo2-E1 rings accurately reproduced the bridging phenotype (Laplante et al., 2016). Simulations also reproduced the bridging phenotype in rings with the ADF/cofilin mutants. Impaired cofilin-mediated severing increased actin filament lengths, thus increasing the ring tension and hence the Laplace forces. Bridging occurred when Laplace forces exceeded the Myo2-actin unbinding threshold. The filament tension was proportional to its length, as longer filaments were pulled by more myosin-II heads. In ADF/cofilin mutants filaments grew ~3-fold longer and had ~ 3-fold normal tension. Bridges peeled away from the plasma membrane at locations where inward Laplace forces exceeded the local anchoring force from Myo2, so bridging was most likely in regions with low Myo2 density, as observed (Cheffings et al., 2019).

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Roles of furrow-associated microtubules and non-centrosomal MTOC during cytokinesis in Chlamydomonas

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Tubulin and related proteins are involved in cytokinesis in a variety of organisms, from the FtsZ ring in bacteria and archaea, the phragmoplast in plants, to the furrow-associated microtubule (MT) array in animals. However, the evolution and conservation of their roles - particularly in cleavage-furrow formation - remain unclear. The green alga Chlamydomonas reinhardtii is phylogenetically close to plants yet divides like animals by cleavage-furrow formation, and this process requires MTs but not F-actin or myosin, similar to those in many other eukaryotes that do not possess a canonical contractile actomyosin ring. Thus, studies of MT roles in Chlamydomonas should shed light on the evolution of cytokinesis in eukaryotes from their early ancestors, which clearly did not involve an actomyosin ring, and the roles of MT and tubulin (-like) proteins during this process. It has been known that Chlamydomonas cells have two types of MT structures: the dynamic cortical MTs and the stable striated rootlet MTs. Through time-lapse imaging of EB1-mNG that tracks the growing tips of MTs, we found that dynamic cortical MT arrays nucleate out of the division plane in cells before and after mitosis, suggesting that there is a non-centrosomal microtubule-organization center (ncMTOC) in this region. The dynamics of these MT arrays is regulated by the cell cycle, because they are suppressed in mutants of APC but not of cyclin B. Consistently with these observations, the minus-end-directed kinesin KCBP localizes to two of the four rootlets that are aligned with the division plane, and this localization is maintained throughout cell division. Because plant KCBPs show similar localization patterns and have been implicated in cytokinesis, our results suggest a functional conservation of this protein family despite the difference in the mode of cell division, i.e., cleavage-furrow formation and cell-plate expansion. Results of our ongoing attempts to elucidate the regulation of MT dynamics and their roles in cleavage-furrow formation through genetic and pharmacological perturbations of MT functions and identification of novel components by proteomics will be discussed.
Molecular organization of Myo2p in cytokinesis node predicts the constriction rate of the contractile ring

How the contractile ring constricts during cytokinesis likely depends on the molecular organization of its proteins. We combined single molecule localization microscopy and confocal microscopy in live cells to elucidate the molecular organization of the contractile ring at the nanoscale and relate it to its constriction rate. Wild-type fission yeast cells assemble their contractile ring by the coalescence of a band of cortical complexes of cytokinesis proteins called nodes. The IQGAP/Rng2p, F-BAR/Cdc15p, formin/Cdc12p and the tips of myosin II/Myo2p tails form the core of the nodes close to the plasma membrane whereas the Myo2p motor-heads spread into the cytoplasm as an inverted bouquet. Cells without Anillin/Mid1p lack visible nodes by confocal microscopy yet assemble contractile rings competent for constriction from the looping of strands of cytokinesis proteins. We leveraged the ∆mid1 contractile ring assembly mechanism to determine how two completely different molecular organizations, nodes versus strands, can yield functional contractile rings. Contrary to previous interpretations, we found that cytokinesis proteins assemble into nodes that align onto actin filaments resulting in strands in ∆mid1 cells indicating that Mid1p is dispensable for node formation. We identified two distinct types of strands in ∆mid1 cells, nascent and enduring. Enduring strands build contractile rings that constrict slower than wild type whereas rings built from nascent strands constrict faster. The Myo2p heads are packed tightly in the nodes of enduring strands, while the Myo2p heads in nascent strands have a broader distribution. Importantly, the organization of the Myo2p heads in the nodes of the strands correlates in a predictive manner with the constriction rate of the contractile ring. Our results suggest that the binding of Myo2p heads to actin filaments cause a tightening of their distribution as depolymerizing the actin causes the largest relaxation in the distribution of the Myo2p heads. Mutations that mimic the tight distribution of the Myo2p heads, seen in the enduring strands of ∆mid1 cells, also recapitulate the slow constriction rate further supporting the association between the molecular organization of the node and constriction rate. Our work is the first to establish a relationship between the molecular organization of nodes and the function of the contractile ring.

Laser microsurgery uncovers the mechanical properties of the constricting contractile ring in fission yeast
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Anchoring the contractile ring to the plasma membrane during cytokinesis is essential for tension generation. Understanding how the contractile ring generates tension remains challenging due to the experimental difficulties of probing forces in live cells. Here, we use laser microsurgery in fission yeast cells to cut the contractile ring and measure the kinetics of the recoiling severed ends to shed light on the mechanics of anchoring. We severed rings from both wild-type cells and cells with depleted F-
BAR/Cdc15p, a putative anchoring protein. After laser microsurgery of contractile rings from wild-type cells, the severed ends recoiled away from each other, stopped and the cut eventually healed. From the viscoelastic profile of recoil kinetics, we built a new mechanical model that predicts that weakening anchoring would decrease the effective elastic contributions to the net force, therefore, increasing the viscoelastic time and amplitude of the recoil. To test our model, we tracked the recoil profile of severed rings in Cdc15p depleted cells. Laser microsurgery of contractile rings in cells depleted for Cdc15p shows that the severed ends of the contractile ring recoiled continuously until they moved out of the imaging plane and they rarely healed. We measured higher viscoelastic time and amplitude of recoil in Cdc15p depleted cells compared to wild-type cells as predicted by our model. These results support that Cdc15p is part of the complex mechanism of contractile ring anchoring. The localization of the myosin II/Myp2p along the length of the ring likely depends on the tension forces distribution inside the ring. We predict that after laser microsurgery, the fluorescence distribution of mEGFP-Myp2p in the contractile ring will be altered due to tension release such that, anchoring sites along the length of the severed contractile will become highly tensed. Upon laser microsurgery, robust caps of mEGFP-Myp2p formed near the edge of the cut tip in wild-type cells suggesting a stable accumulation of the protein due to localized tension. Similar caps of mEGFP-Myp2p formed in Cdc15 depleted contractile rings but rapidly decayed as the severed segment fell out of the fixed imaging plane. Our work is the first to establish a relationship between protein distribution and mechanical functions and reveal the role of Cdc15p in the complex anchoring mechanism.

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Two putative transient receptor potential channels Pkd2p and Trp1322p regulate cytokinetic calcium spikes of fission yeast

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During cytokinesis of animal embryos, the intracellular calcium concentration increases transiently at the cleavage furrow. Recently, our group discovered that similar calcium spikes accompany the cleavage furrow ingestion and the cell separation of the unicellular model organism fission yeast Schizosaccharomyces pombe. However, the molecular mechanism of these calcium spikes remains unknown. Here we examined whether any putative ion channels contribute to these calcium spikes during cytokinesis. We quantified the calcium spikes using a genetically encoded calcium indicator GCaMP in seven ion channel mutants respectively. We identified two ion channel mutants that altered the calcium spikes during cytokinesis. First, Pkd2p is an essential fission yeast homologue of polycystin channel that localizes to the cleavage furrow during cytokinesis. A depletion mutant of Pkd2p (pkd2-81KD) increased the amplitude of the calcium spikes during the furrow ingestion by 50%. However, the mutant reduced the amplitude of the separating spikes by 30%. Further, the contractile ring of pkd2-81KD constricted at an increased rate but the cell separation failed frequently. Consistent with the importance of Pkd2p in regulating the cytokinetic calcium spikes, the pkd2 mutant is hyper-sensitive to the depletion of extracellular calcium. Secondly, Trp1322p, a non-essential transient receptor potential (TRP) channel localized to the plasma membrane. A deletion mutant of trp1322 reduced the amplitude of the separating spikes significantly but didn’t alter the constricting spikes. Unlike the pkd2 mutant, the trp1322 mutant did not exhibited cytokinesis defect or hyper-sensitivity to the depletion of external calcium. We concluded that these two fission yeast TRP channels, Pkd2p and Trp1322p contribute to the regulation of the cytokinetic calcium spikes.
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**Writhing of cytokinetic contractile rings reveals that the contractile ring is an elastoporous cable**

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We report extraordinary writhing dynamics of isolated fission yeast contractile rings, revealing basic material properties of the actomyosin ring normally hidden under physiological circumstances. Contractile rings exhibited cable-like writhing and coiling, with repeated twisting of the rings leading to multiple coils, similar to the twisting of a telephone cable. Our results suggest the twisting torque is produced by myosin-II in the ring that twisted around anchored actin filaments, and the twisting rates we measured are very close to those previously reported for myosin II-actin twisting from *in vitro* gliding assays (Nishizaka et al., 1993; Beausang et al., 2008). We show the twisting is quantitatively as would be predicted for a solid cable, and from the measured writhe versus ring length we extracted the effective ratio of ring twisting and bending moduli. A mathematical model quantitatively reproduced the cable-like twisting behavior, based on a continuum treatment of the contractile ring as an elastoporous solid, similar to an emerging view of the cell cytoplasm (Moeendarbary et al., 2013). The model predicts that after a short transient the ring deforms affinely, with constant twisting rate like a solid cable whose mechanical rigidity is set by its twisted actin filaments. Thus, our results show that the contractile ring, considered as a whole, is an elastoporous cable consisting of actin filaments and an effective fluid of myosin-II. The elastoporous ring cable is highly anisotropic, with anisotropic twisting properties along and perpendicular to its length. We observed these writhing rings in fission yeast cell ghosts, obtained by cell wall digestion followed by membrane permeabilization that releases cytoplasm while leaving membrane-anchored contractile rings behind (Mishra et al., 2013). The cell ghost is a laboratory allowing study of contractile rings in extraordinary and revealing circumstances (Wang and O'Shaughnessy, 2019). Writhing of rings occurred when entire sections of contractile rings become unanchored from the weakened membrane, leaving a short anchored section. The unanchored segment shortened and writhed, due to an apparent twisting torque at the anchoring points. Repeated rotation produced multiple coils in the rings. Our analysis revealing that the ring writhes as a solid cable was enabled by image analysis, which revealed 3D ring contours from which we found that the dependence of writhe on ring length was that of a solid cable with twisting to bending moduli ratio ~4, similar to the ratio from previous measurements of single actin filaments. The ring twisting rate was ~0.3 revs per µm shortened, consistent with the 0.25-2 revs per µm measured for myosin II twisting around actin filaments (Nishizaka et al., 1993; Beausang et al., 2008).

P992

**The number of cytokinesis nodes scales with cell size in fission yeast.**

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Cytokinesis nodes are discrete structural units with stoichiometric ratios and distinct distributions of constituent proteins, essential to make the contractile ring during cytokinesis in fission yeast. These nodes appear during interphase in a broad band on the inner surface of the plasma membrane around
the middle of the cell. It is not known how cells set the number of nodes. FPALM super resolution microscopy resolves each node but cannot collect a 3D image of the whole equator of the cell. Confocal microscopy can image the whole cell but cannot resolve closely spaced nodes, so the total number of nodes is uncertain. Here we used Airyscan confocal microscopy to measure the number of cytokinesis nodes in fission yeast cells with a range of sizes. Airyscan has a resolution of ~140 nm, good enough to resolve a large population of dim nodes and is capable of 3D reconstructions of whole cells. We imaged wild type (WT control), short (wee1- 50 mutant), long (cdc25-22 mutant), thick (Rga4Δ mutant) and thin (Rga2Δ mutant) cells expressing the node marker Blt1p-mEGFP. We divided the total fluorescence of the whole broad band of nodes or contractile ring by the average total fluorescence of individual unitary nodes to estimate the number of nodes in the broadband or contractile ring in a cell. Wild type fission yeast cells make about 200 cytokinesis nodes located in a broad band around the middle of the cell. Most, but not all of these nodes condense into a contractile ring. Pom1 kinase helps to restrict these nodes to the middle of the cell but does not control their number. Cytokinesis node number scales with cell size except in rga4Δ mutant cells, which forms fewer cytokinesis nodes than WT cells despite having similar volume and surface area.

P993

A novel protein - SABRE that regulates cell division and growth through the endoplasmic reticulum
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SABRE is a large protein that was original identified in plants and implicated in functions involving cell expansion, division plane orientation and planar polarity. In the moss Physcomitrium (Physcomitrella) patens, we generated a null mutant of the single SABRE gene. Phenotypic analyses demonstrated that both polarized growth and diffuse growth were inhibited. In the filamentous tissue, known as protonemata, cells expand only at the apical cell tip and divide once they reach a certain length. In ∆sabre, during cell division we observed delays in cell plate formation and sometimes catastrophic failures with unknown brown material accumulating at the position of the new cell plate and often cell death. We generated a functional 3XmNeonGreen tag at the C terminus of SABRE, and determined that SABRE formed dynamic puncta that co-localized with regions of the endoplasmic reticulum (ER) both in the cytoplasm during interphase and at the new cell plate during division. Loss of function in sabre causes abnormal ER morphology, characterized by large aggregates in the cytoplasm. During cell division, the SABRE and ER signals intensified during cell plate maturation, consistent with the timing of the onset of cell plate formation failure in cells lacking SABRE. At this time, for cells that managed to divide in ∆sabre cells, the ER at the cell plate buckled. Furthermore, we observed exaggerated basal movement of the nucleus in apical cells in ∆sabre, as revealed by another continuous compartment of the ER - the nuclear envelope. Together, we discovered that SABRE associates with the regions of the ER, and regulates cell division and polarity by influencing ER morphology and dynamics.
Cytoskeletal Regulators of Development

P994

Local Mechanical Forces Regulate Global Morphogenetic Patterning in the Developing Mouse Mammary Gland

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Mammary epithelial branching morphogenesis is a stochastic process wherein the epithelium recursively elongates and bifurcates as it extends collectively into the fat pad to produce a branched epithelial tree. While the final geometry of the mammary epithelium is random, the developing epithelial tree is largely oriented along the long axis of the gland. How a stochastic branching program can predictably orient the developing mammary epithelium has remained a long-standing mystery. Here, we combined experimental analysis of pubertal mouse mammary glands and a 3D-printed engineered tissue model with computational analysis of morphogenesis to investigate the origin and the dynamics of the epithelial orientation-bias during pubertal mammary epithelial development. Confocal microscopy analysis revealed that the bias in epithelial orientation emerges in 4-week-old glands and is maintained throughout puberty until the widespread formation of lateral branches. In contrast to existing hypotheses, we observe that the epithelial orientation-bias emerges in the absence of type I collagen fiber alignment within the fat pad. Using branching and annihilating random walk simulations, we find that the bifurcation angle of terminal end buds regulates both the dynamics and the extent of the epithelial orientation-bias. Simulations of epithelial bifurcation using a finite element method-based model suggest that mechanical forces derived from the local accumulation of extracellular matrix are sufficient to regulate the bifurcation angle of terminal end buds. Our results indicate that local mechanical forces dictate the relative positioning of daughter branches and regulate the global bias in mammary epithelial orientation in the absence of global signaling gradients. These findings show that local signals can generate global order, and may have implications for understanding the global patterning of other branched epithelial tissues.

P995

Golgi proteins Arf1 and Sec71 govern neural stem cell polarity through non-muscle myosin II

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Understanding how neural stem cells divide asymmetrically is central for stem cell and cancer biology. *Drosophila* neural stem cells (NSC), or neuroblasts, are an excellent model for understanding stem cell asymmetry and homeostasis. Each neuroblast divides asymmetrically to give rise to a self-renewing neuroblast and a neural progenitor that generates post-mitotic neurons. The perturbation of asymmetric division can lead to defects in brain development and homeostasis. Mutations in critical Golgi proteins such as ARF1 and Sec71/ARFGEF2 cause malformations of cortical development, a type of neurodevelopmental disorder in humans. Furthermore, mutations in human Sec71/BIG2 (ARFGEF2 – HGNC) lead to autosomal recessive periventricular heterotopia with microcephaly (ARPHM), a brain disorder characterized by defective neural proliferation and migration. However, the role of Arf1 and its guanine-nucleotide exchange factor (GEF) ARFGEF2/Sec71 in neuroblast polarity and brain development
is unknown. Here we show a new role for *Drosophila* Arf1/Arf79F and its GEF Sec71 in asymmetric cell division and homeostasis of neuroblasts likely through its interaction with non-muscle myosin II regulatory light chain (RLC), spaghetti squash (Sqh). Loss of Arf1 and Sec71 caused Sqh delocalization from the cell cortex and asymmetric division defects. In addition to its effect on myosin, we also find that Arf1 and Sec71 regulate Phosphatidylinositol 4-phosphate (PI(4)P) localization to the plasma membrane in neuroblasts suggesting that Arf1 may regulate myosin II and phosphatidylinositol lipids to control asymmetric division of neural stem cells.

P996

**Sonic Hedgehog Signaling Patterns Apical Constriction During Cranial Neural Tube Closure**

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The development of multicellular tissues requires the precise interpretation of positional information by cells. Disruption of this process can have severe developmental consequences. Failures in neural tube closure are among the most common birth defects, impacting 1:1,000 pregnancies worldwide. Exencephaly—a failure to close the presumptive brain—accounts for approximately one-third of neural tube defects. Over 200 mutant mouse strains exhibit exencephaly, but the cellular mechanisms that drive cranial closure remain opaque. Using high-resolution microscopy and quantitative image analysis, we identified a spatially regulated pattern of apical constriction in the cranial neural tube. We show that cranial closure is initiated by the sustained apical constriction of a large population of thousands of cells located lateral to the midline, while midline cells remain static. Loss of Ift122 or Ttc21b, two members of the conserved intraflagellar transport A (IFT-A) complex that regulates cilia function and Sonic hedgehog (Shh) signaling, disrupts patterned cell remodeling in the cranial neural plate, resulting in highly penetrant exencephaly. These mutants exhibit a failure of apical constriction associated with defects in apical actomyosin organization in lateral cells, resulting in a failure of the cranial neural plate to convert from convex to concave. These defects are due to a dysregulated pattern of Shh signaling, as activation of the Shh signaling response throughout the midbrain neuroepithelium recapitulates the exencephaly defects of IFT-A mutants. This work reveals a novel pattern of spatially regulated apical constriction behaviors that drives cranial neural tube closure and uncovers a key source of positional information governing this pattern.

P997

**The function of Scribble in neural convergent extension**

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Neural tube defects (NTD) occur in 1:500 pregnancies and result in severe birth defects. Neural tube closure (NTC) in vertebrates relies on the elongation, driven by convergent extension (CE), and bending of the epithelium through cell wedging and apical constriction. NTC is dependent on several cellular processes including polarized cell intercalation, cell shape changes, and cytoskeletal dynamics and loss of proteins that regulate these functions results in craniorachischisis (CRN). One such protein is Scribble (Scrib) which is best known for its roles in regulating apical-basal polarity and apical junctional
organization. *Scrib* mutants display short body axis phenotypes, axial torsion, and CRN, although how Scrib regulates neural cell behavior during NTC remains unknown. We examined this in *Scrib* mice, using live imaging of cell behavior in the neural plate of E8.0 embryos. We find that the *Scrib* mutation inhibits overall tissue shape changes, leading to decreased CE. Analysis of cell intercalation and cell shape revealed that *Scrib* leads to defects in polarized cell intercalation as well as loss of both apical constriction and cell wedging. Interestingly, we also observed that mutant embryos exhibit a change in the frequency of rosette resolution as a mechanism for cell rearrangement. *Scrib* mutant embryos exhibit mislocalized actin and myosin, and live imaging of embryos expressing GFP-Lifeact shows alterations in the dynamics of apical actin in mutant embryos. In addition, loss of Scrib localization leads to specific effects on tight junctions, with effects on apical localization of the junctional proteins ZO-1 and Par3. This work is supported by the Eunice Kennedy Shriver National Institute of Child Health and Human Development-R01HD087093.

P998

**Dissecting the mechanisms that orchestrate myosin patterns in developing tissues using optogenetics**

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Epithelial tissue sheets can be shaped into complex forms through series of stretching, flowing, and folding events - orchestrated by sequences of myosin-generated forces that are patterned in space and time. Some myosin II patterns are highly conserved and used repeatedly in development, including the planar polarized patterns that narrow and elongate tissues during convergent extension. Yet, the mechanisms that establish and maintain these patterns or instead convert them into new patterns over the course of development are not well understood. In *Drosophila*, a planar polarized pattern of myosin II regulated by the conserved Rho/Rho-kinase signaling pathway is required for convergent extension during body axis elongation. Here, we use optogenetics to manipulate RhoGEF (optoGEF) and RhoGAP (optoGAP) patterns in the germband epithelium and analyze the effects on myosin dynamics, mechanical tension, cell intercalation, and convergent extension during *Drosophila* axis elongation. We find that optogenetic recruitment of optoGEF at the apical cell membrane is sufficient to rapidly increase medial myosin accumulation, followed by an unexpected and more gradual decrease in junctional myosin, transforming the planar polarized myosin pattern into one reminiscent of the radial patterns observed in some epithelia prior to invagination. This transition is associated with increased and more isotropic tissue retraction after cutting the tissue using laser ablation, indicating that changes in the myosin pattern are translated into altered force distributions in the tissue. In contrast, optogenetic recruitment of optoGAP decreases junctional and medial myosin accumulation and reduces tissue retraction following laser ablation. Uniform recruitment of either optoGEF or optoGAP across the tissue nearly abolishes myosin planar polarity and reduces convergent extension, but these perturbations have distinct effects on the cell behaviors that drive tissue movements, revealing the requirement for spatially patterned Rho signaling in myosin planar polarity and cell intercalation. These studies reveal the dynamic transition between two distinct myosin patterns, providing new insights into the mechanisms that establish, maintain, and remodel myosin patterns during development, and directly link patterns of Rho signaling to the myosin-generated force patterns that direct epithelial morphogenesis.
Epithelial Geometry Controls Paracrime Signaling to Shape Supporting Vasculature
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Epithelia possess undulations that serve as stem cell niches. Curved surfaces within intestinal villi, epidermal ridges and kidney papillae also possess defined spatial relationships with supporting vasculature. Our current work aims to identify molecular and physical mechanisms by which epithelial topography guides vascularization. Controlling keratinocyte cell area by targeting the cytoskeletal regulator, Dia1, or by forced crowding altered transcriptional programs relevant to stem cell fate and differentiation. Amongst the responsive genes were soluble chemokines, morphogens and growth factors. Regulators of angiogenesis, in particular, responded both negatively and positively to the reduced cell area caused by reintroducing Dia1. This set of area sensitive factors included FGF2, IGF2, CXCL14 and BMP7, the latter two being known targets of p63, a transcription factor critical to determining epidermal cell fate. To test whether an altered secreted protein repertoire might have physiological consequences, we plated Dia1-deficient and Dia1-rescue keratinocytes atop collagen gels containing endothelial cell aggregates. Dia1-deficiency in the epithelial compartment substantially enhanced growth and branching of endothelial aggregates compared to Dia1-rescue and epithelia-free controls. Currently, we are investigating whether these results inform the behavior of cells in the more physiological, 3D regime of epithelial undulations. The goal is to test the hypothesis that undulations cause heterogenous expression patterns of key soluble factors within epithelia, resulting in the guided growth and sculpting of microvasculature.

Investigating the formation of spatial and directional patterns in the mammalian epidermis
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During embryonic development, spatially-patterned morphogen signals direct the cellular rearrangements that shape tissues and organs. Hair placodes of the developing murine epidermis comprise an ideal model system to investigate how signaling pathways and cell fate specification drive morphogenetic movements. Hair placodes are repeated across the skin surface in a spatially-defined pattern and they undergo highly stereotyped movements as they bud from the epithelium. We previously showed these movements are organized in a counter-rotational pattern, a process of collective motility requiring myosin, Rho kinase, and planar cell polarity. Because the signaling pathways that specify placode fate are well-defined, we set out to determine the contribution of each distinct cell type in placode morphogenesis and to define the downstream transcriptional changes that contribute to counter-rotational movements. Hair placodes are composed of three distinct cell types: two epithelial cell fates that we refer to as inner and outer cells, as well as an underlying dermal population, the dermal condensate. Here we show that Wnt signaling is necessary and sufficient to specify inner cells and, through epidermal Shh signaling, to induce outer cell fate. We found that in the absence of outer cells, placodes fail rearrange in a counter-rotational pattern suggesting that outer cells provide a compartment within which inner cells can rearrange. We investigated how differences in cell adhesion might contribute to the formation of this compartment and found that E- and P-Cadherin are expressed in inversely-oriented gradients that emanate outward from the placode center. By flattening E- and P-
Cadherin gradients across the epithelium, we are currently investigating the role of these adhesion gradients in placode morphogenesis. Our results show how upstream signaling pathways lead to the downstream cellular changes that are ultimately linked to morphogenesis. This work was supported by the NIH-NIAMS under award number F31-AR074246 to L.L.; R01-AR066070 to D.D. B.T. was supported by NIH-NIGMS under award number T32-GM7388. R.S. was supported by the NSF-GFRP.

P1001

**A Novel Role for KIF17 in the Developing Cerebellum**

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Since its initial discovery in *Drosophila* nearly forty years ago, the Hedgehog (HH) signaling pathway has been demonstrated to direct the development and maintenance of nearly every organ system across invertebrate, vertebrate, and mammalian animal models. A key organelle that regulates HH signaling is the primary cilium, a microtubule-based projection from the cell membrane that serves as signaling centers for multiple pathways. Mammalian HH signaling requires functional primary cilia, as ciliary trafficking is required for proper GLI processing, including phosphorylation and cleavage. Kinesin-2 motor proteins mediate anterograde transport of cargo through primary cilia. There are three motor complexes in the Kinesin-2 family: heterodimeric motors KIF3A/KIF3B and KIF3A/KIF3C, and homodimeric KIF17. KIF3A/KIF3B is required for ciliogenesis, while KIF17 and KIF3C are less well studied in vertebrate ciliogenesis. Using a *Kif17lacZ* allele, *Kif17* expression is observed in the developing cerebellum, within Purkinje cells (the source of SHH) and HH-responsive cerebellar granule neural progenitors (CGNPs). Further, the loss of KIF17 in the cerebellum, a tissue that requires HH signaling for normal patterning and proliferation, leads to cerebellar hypoplasia due to decreased proliferation of CGNPs and decreased expression of HH target genes across the cerebellum. Confoundingly, when *Kif17*−/− CGNPs are cultured *in vitro*, these cells display increased proliferation, even in the absence of growth factor stimulation. Further, reduced HH target gene expression in cells that do not express *Kif17* suggests that the defect in the cerebellum lies upstream in the HH pathway within the Purkinje cells, the source of Sonic HH ligand. Despite an increase in overall *Shh* mRNA, I observe a decrease in processed SHH protein in *Kif17*−/− cerebella. Furthermore, mice with conditional deletion of *Kif17* within Purkinje cells phenocopy *Kif17lacZlacZ* mice. Overall, these data suggest a novel role for KIF17 in Purkinje cells in mediating levels of SHH ligand during cerebellar development.

P1002

**Regeneration of the epithelial structure in neural stem cells during mammalian brain development**

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Neural stem cells, also known as radial glia (RG), maintain the epithelial structure during the mammalian cortical development. The spindle orientations in RG divisions are nearly planar to the ventricular surface, and thus the cleavage plane mostly bisects the apical surface, enabling both daughter cells to inherit the apical structure. The spindle orientation of RG is regulated by a dynein-anchoring complex containing LGN. The prevailing view claims that at the early proliferative stage, symmetric divisions of RG require tight regulation of the spindle orientation, the perturbation of which causes precocious neurogenesis and apoptosis. By contrast, our previous studies indicated that at the subsequent neurogenic stage, spindle misorientations via LGN disruption mainly affect the migratory state and position of RG, rather than the daughter cell fates. LGN disruption in mice causes the cleavage plane to frequently bypass the apical surface, which results in the loss of the apical endfoot in one daughter cell. These daughter cells translocate to the basal side while maintaining the self-renewability and become outer/basal RG (oRG), a hallmark of gyrencephalic development. Thus, at the neurogenic stage, spindle orientation is essential for maintaining the RG epithelial structure but not for daughter cell fates or survival. Why RG show such differential properties depending on the stage is unsolved. Here, we show that RG at the proliferative stage are highly robust against perturbation due to a regeneration ability of the epithelial structure but not a strict spindle orientation. At the proliferative stage in the LGN-mutant mice, spindle misorientations and consequent loss of the apical endfoot occurred as frequently as at the neurogenic stage. However, we observed neither precocious neurogenesis nor apoptosis at the proliferative stage. Instead, we found that the daughter cells missing the apical endfoot regenerated it, which led to recovery of the entire epithelial structure, and became RG. These observations contradict the conventional notion. Regenerating endfeet attached to surrounding cells with ectopic adherens junctions at their leading edge. This ability gradually declined during neurogenesis, which allows RG missing the apical endfoot to transform into oRG at the late neurogenic stage. The regeneration ability was mediated by Notch-R-Ras-integrin beta1 pathway, activity of which also declined during neurogenesis. Overactivation of this pathway in ferret, a gyrencephalic model animal, oRG formation was impaired. Thus, our study reveals a temporally changing cryptic property, which initially ensures symmetric divisions of RG and subsequently provides a basis for the formation of the new germinal layer in the mammalian brain development.

P1003

Actin based protrusions interact with tricellular junctions to guide single cell intercalation within mucociliary epithelium

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During development tissues evolve into more complex entities by combining different cell types, which may require cells to move between their neighbors in order to reach the positions where they ultimately exert their function. This process is additionally required for tissue maintenance and the renewal of specialized cells in an epithelium and can also be related to how cancer cells navigate through healthy tissues. Our work aims to understand the central molecular players responsible for the movement and positioning of specialized epithelial cells as they integrate the mucociliary epithelium of the Xenopus embryo. Our results show that the intercalation of new specialized multiciliated cells (MCCs) is guided by the extension of actin-based protrusions at the epithelial tricellular junctions established by the overlaying goblet cells. We have also determined that LSR, a known epithelial tricellular junction protein,
is involved in the movement of the intercalating cells, suggesting that it transmits information between the MCCs and goblet cells. Interestingly, epithelial tricellular junctions play wide-ranging key roles in many physiological processes, from homeostasis to infection and cancer dissemination. However, their impact in individual cell movement is yet to be defined. Our work helps define these structures as novel regulators of cell movement and cell-cell communication.

**Dynein: Regulation**

P1004

**The MAP She1 coordinates dynein-mediated spindle positioning by spatially restricting dynein activity in yeast**

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During cell division in budding yeast, the mitotic spindle is actively positioned within the bud neck by cytoplasmic dynein prior to anaphase onset and cytokinesis. Cortically-anchored dynein motors walk along astral microtubules that emanate from the spindle pole bodies, thereby pulling the spindle and nucleus into the narrow bud neck connecting mother and daughter cells: the future site of cytokinesis. In order to perform this function with spatial and temporal precision, dynein is assisted by numerous factors including the microtubule-associated protein (MAP) She1. Our recent in vitro data demonstrated that She1 affects dynein motility by simultaneously interacting with the dynein microtubule binding domain and the microtubule, and our prior in vivo data revealed that She1 assists dynein-mediated spindle positioning by affecting the ability of dynein to move the spindle across the bud neck. However, a complete model that reconciles these in vitro and in vivo data is lacking, as is a comprehensive understanding of the role of She1 in affecting dynein activity in vivo. Here, we performed a detailed analysis of dynein-mediated spindle movements in live cells, which revealed that She1 assists dynein-mediated spindle positioning by ensuring that dynein maintains the spindle in close proximity to the bud neck. We find that this is a consequence of She1 specifically attenuating the initiation of dynein-dynactin-mediated spindle movements in the mother, but not the bud cell. Thus, She1 promotes proper spindle positioning by ensuring dynein-mediated spindle movement events are polarized toward the daughter cell. We find that this process depends on She1 binding to astral microtubules, and not spindle microtubules where She1 also localizes. Our data also indicate that She1 attenuates cellular dynein activity in a manner that requires its interaction with dynein’s microtubule binding domain. Although the manner by which She1 discriminately affects dynein activity in the mother and daughter cells is not entirely clear, our findings suggest it may do so by exhibiting a strong localization bias to the mother cell. In summary, our findings indicate that She1 regulates dynein activity by ensuring biased movement of the spindle towards the bud neck during dynein-mediated spindle events.
P1005

Cargo recognition by the dynein adaptor Bicaudal D2

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The dynein adaptor Bicaudal D2 (BicD2) recognizes cargo for dynein-dependent transport and links them to the motor complex, but the molecular mechanism for cargo selection by BicD2 is unknown. Here, in order to obtain mechanistic insights into how BicD2 selects cargo, we have characterized the interaction between cargo adaptor Nup358, which is a protein subunit of the nuclear pore complex and BicD2, by combining mutagenesis and biophysical approaches. We have identified several point mutations that strongly diminish the interaction between Nup358 and BicD2, which are suitable for studies of these pathways in the context of cells. Our results provide insights into the molecular mechanism of cargo selection of BicD2, which facilitates transport pathways that are important for brain development and faithful chromosome segregation.

P1006

Autophagosomal maturity coordinates dynein effector scaffolding in axons


Autophagy is a degradative pathway required for neuronal homeostasis; autophagy defects are observed in neurodegenerative diseases including Parkinson’s and Huntington’s disease. In neurons, autophagosomes form constitutively at the axon terminal and mature by fusing with lysosomes during dynein-mediated transport to the soma. However, it is unknown how the dynein-autophagosome interaction is regulated during this maturation. We now identify a series of handoffs between dynein effectors as autophagosomes transit along the axons of primary murine hippocampal neurons. Through both live-cell imaging and proximity ligation assays, we find the scaffold protein JIP1 is significantly enriched on nascent autophagosomes in the distal axon, where it interacts with the dynein complex and is required to initiate the long-range transport of autophagosomes. The transit of autophagosomes along the axon shaft requires neurodegeneration-associated protein Huntingtin and its interacting partner HAP1. In vitro binding and single molecule motility assays demonstrate that HAP1 induces microtubule binding of the dynein-dynactin complex and activates dynein-driven motility. HAP1 interacts with components of dynein and dynactin via both canonical and noncanonical interaction sites. Point mutations in HAP1 that disrupt these binding sites have a dominant negative effect on autophagosomal transport. Surprisingly, we find that in addition to HAP1, the lysosomal motor scaffold JIP3 is also associated with most axonal autophagosomes. However, JIP3 specifically regulates the transport of mature, acidified autolysosomes in the proximal axon, within 200 µm of the soma. Inhibiting autophagosomal transport is known to disrupt their maturation; we now find that inhibiting autophagosomal maturation pharmacologically via Bafilomycin A1 treatment or genetically via a dominant negative syntaxin-17 mutant disrupts the recruitment and activity of dynein effectors. Thus maturation state and cofactor activation are tightly linked. Collectively, these results describe a novel
maturation-based motor effector handoff on neuronal autophagosomes key to autophagosomal motility, cargo degradation, and ultimately axonal health. *Supported by NIH grant R35 GM126950.*

P1007

**Dynein adaptors such as Drosophila Bicaudal (DmBicD) recognize cargo and are required to activate dynein for processive transport.**

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In the absence of cargo, auto-inhibited BicD cannot recruit dynein, but the underlying mechanism of cargo-induced activation is elusive. Our single molecule processivity assays shows that auto-inhibition is abolished in the full-length Bicaudal mutant F684I, which activates dynein motility even in the absence of cargo. To investigate the structural basis for activation, we determined the X-ray structure of the C-terminal cargo-binding domain (CTD) of Dm BicD-CTD/F684I at 2.4 Å resolution. The structure revealed that the mutant has a homotypic coiled-coil registry, in which the two helices of the homodimer are aligned, whereas the wild-type structure has an asymmetric registry, in which the two chains are vertically shifted by ~1 helical turn in a portion of the coiled-coil. In the mutant, N-terminal ~20-residue region is disordered for one of the two chains. MD simulation and CD spectroscopy data suggested the structural flexibility of N-terminal region of Dm BicD-CTD/F684I and confirmed the intact protein is in the crystal and alpha-helical. We propose that a coiled-coil registry shift activates BicD for dynein recruitment. The registry shift could either be induced by the F684I mutation or by cargo-binding. Also, the human homolog of the BicD-CTD/F684I mutant (BicD2-CTD/F743I) showed diminished binding to its cargo Nup358. Thus, we propose that in mammalia, a coiled-coil registry shift modulates cargo selection for BicD2-dependent transport pathways, which are important for brain development and chromosome segregation. Furthermore, we investigated the mechanism for cargo recognition by BicD2. Our data from circular dicroism spectroscopy and small-angle X-ray scattering suggest that the BicD2 binding domain of Nup358, which is intrinsically disordered on its own, takes on a helical structure when binding to BicD2. This alpha-helix of Nup358 could be a structural feature for cargo recognition by BicD2.

P1008

**Bicd2 control of Nesprin 2-mediated Dynein recruitment to the Nuclear Envelope in brain and cultured cells.**

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Microtubule motor-based nucleus and centrosome transport play critical roles in brain development. During G2-dependent apical nuclear migration in radial glial progenitor cells (RGPs) cytoplasmic Dynein is initially recruited to the Nuclear envelope NE by the nucleoporin RanBP2 through the adaptor protein BicD2 (Hu et al, 2013). Migration of post mitotic neurons towards the cortical plate (CP) also requires dynein-mediated nuclear transport. It has been reported that Nesprin-2 recruits motor proteins to the NE in non-neuronal cells. Our objective in this study was to determine the mechanisms controlling this aspect of Nesprin 2 behavior. To this end, we expressed functionally distinct fragments of the ~800 kDa Nesprin 2 protein in E16 rat brain using *in utero* electroporation. We found by fixed and live imaging in brain slices that deletion of the motor binding region of Nesprin 2 causes severe reduction in neuronal
migration to the CP. Centrosome advance was unaffected, resulting in nucleus-centrosome dissociation by as much as 100 μm and more. Surprisingly, Nesprin-2 recruited dynein to the NE indirectly via a novel Nesprin-2 interaction with the dynein adaptor BicD2. In this regard Nesprin 2 acts during G0 similarly to another BicD2 interactor and G2 dynein recruitment factor, RanBP2. To test whether Nesprin-2 and RanBP2 interact with common or distinct sites within BicD2, we introduced missense mutations in BicD2, and found differential effects on binding of the two BicD2 interactors. How BicD2/dynein recruitment during different stages of the cell cycle and of brain development is controlled have now emerged as important new questions for further research. Supported by NIH grant # 1R01GM132478-01A1

**P1009**

**A coiled-coil registry shift may activate Bicaudal D for dynein recruitment upon cargo binding**

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Key questions in the dynein field are how cargo is recognized by dynein adaptors, such as *Drosophila* Bicaudal D (BicD) and how dynein is activated for processive motility by these adaptors. BicD is auto-inhibited and activates dynein motility only after cargo is bound, but the underlying mechanism is elusive. In contrast, we show that the full-length BicD/F684I mutant activates dynein processivity even in the absence of cargo. Our X-ray structure of the C-terminal domain of the BicD/F684I mutant reveals a coiled-coil registry shift; in the N-terminal region, the two helices of the homodimer are aligned, whereas they are vertically shifted in the wild-type. One chain is partially disordered and this structural flexibility is confirmed by computations, which reveal that the mutant transitions back and forth between the two registries. We propose that a coiled-coil registry shift upon cargo binding activates BicD for dynein recruitment. Moreover, the human homolog BicD2/F743I exhibits diminished binding of cargo adaptor Nup358, implying that a coiled-coil registry shift may be a mechanism to modulate cargo selection for BicD2-dependent transport pathways. This Nup358/BicD2 pathway serves to position the nucleus with respect to the centrosome and is essential for a fundamental process in brain development that is required for radial glial progenitor cells to differentiate, which are precursors for the majority of neurons and glia cells in the vertebrate neocortex.

**P1450**

**The vezatin homolog VezA is a novel dynein regulator in vivo**

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Cytoplasmic dynein binds its cargo via the dynactin complex and cargo adapters, which activate dynein by shifting it from an autoinhibited conformation to a parallel conformation, a process facilitated by LIS1 that prevents the autoinhibited conformation. Here we found VezA/vezatin as another important factor for dynein activation in *Aspergillus nidulans*. Loss of VezA significantly affects the cargo-adapter-driven dynein activation as judged by dynein relocation from the microtubule plus ends to the minus ends. VezA interacts with dynein-dynactin as evidenced by binding of the cytosolic VezA to dynein-dynactin in pull-down assays. Interestingly, while the phi mutation preventing the autoinhibited
conformation of dynein bypasses LIS1 to a significant extent, it does not bypass the requirement of VezA for dynein-mediated early endosome transport. Finally, pulldown assays also suggest that VezA affects the structural integrity of the dynactin complex in a subtle way. Thus, we have identified a novel dynein regulator whose mechanism of action differs from that of LIS1 or a cargo adapter.

**Endocytosis 2**

**P1010**

**Calcineurin-dependent regulation of endocytosis by a plasma membrane ubiquitin ligase adaptor Rcr1**

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Rsp5, the Nedd4 family member in yeast, is an E3 ubiquitin ligase involved in numerous cellular processes, many of which require Rsp5 to interact with PY-motif containing adaptor proteins. To identify novel Rsp5 adaptor proteins, we looked into the yeast proteome for PY-motif containing transmembrane proteins. By employing a combination of genetic, biochemical and cell biological approaches, we show that two paralogous transmembrane Rsp5 adaptors, Rcr1 and Rcr2, are sorted to distinct cellular locations: Rcr1 is a plasma membrane (PM) protein whereas Rcr2 is sorted to the vacuole. Rcr2 is delivered to the vacuole membrane using ubiquitin as a sorting signal. Rcr1 is delivered to the PM by the exomer complex using a newly uncovered PM sorting motif. Further, we show that Rcr1, but not Rcr2, is upregulated via the calcineurin/Crz1 signaling pathway. Upon exogenous calcium treatment, Rcr1 ubiquitinates and downregulates the chitin synthase Chs3. We propose that the PM anchored Rsp5/Rcr1 ubiquitin ligase-adaptor complex can provide an acute response to degrade unwanted proteins under stress conditions, thereby maintaining cell integrity.

**P1011**

**Amino Acids Inhibit Growth Factor-stimulated Macropinocytosis in Macrophages**

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Macropinocytosis is an evolutionarily conserved process by which cells internalize extracellular solutes into large vesicles called macropinosomes. This process is involved in many basic physiological processes such as cell motility, antigen presentation, and cell growth. Many cancer cells use macropinocytosis to obtain nutrients to meet their high metabolic needs. In murine bone marrow-derived macrophages, macropinocytosis can be induced by the growth factor macrophage colony-stimulating factor (M-CSF), bacterial products such as LPS, phorbol myristate acetate (PMA) and various cytokines. To examine whether nutrient supply can modulate rates of macropinocytosis, this study measured the effect of extracellular amino acids on rates of solute accumulation by macropinocytosis in macrophages. We utilized a flow cytometry-based approach to measure the internalization of a high molecular weight fluorescent dextran in buffered saline supplemented with various amino acids. Most essential amino acids suppressed macropinocytosis, whereas most non-essential amino acids did not. Using leucine as representative inducer of this suppression, we investigated the mechanisms of macropinocytosis inhibition. Leucine suppressed uptake by macropinocytosis in response to M-CSF and the cytokine IL-34, both ligands for the M-CSF receptor, but not when macropinocytosis was stimulated by LPS, PMA or the
chemokine CXCL12. Leucine lowered cell surface levels of the M-CSF receptor, which resulted in a
decrease in the size of macropinosomes. These findings demonstrate that suppressive amino acids
decrease macropinosome size and net solute uptake by removal of M-CSF receptors from the
macrophage cell surface. Future studies aim to elucidate the mechanism by which leucine lowers cell
surface levels of the M-CSF receptor.

P1012

Knockdown of ORP9 Delays the Maturation of Tubulobulbar Complexes in the Testis and Leads to
Spermiation Failure
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Tubulobulbar complexes (TBCs) are large, endocytic devices that likely evolved from classic clathrin-
moted endocytosis machinery (CME). Crucially, the release of late spermatids from the seminiferous
epithelium (spermiation) requires the internalization of intercellular junctions by TBCs. While TBCs have
similarities with CME vesicles, there are several important morphological differences. Unlike with
clothrin-coated pits, TBCs form extensive membrane contact sites (MCSs) with the endoplasmic
reticulum (ER). Some of the well-established functions of ER MCSs are calcium and lipid exchange. We
have previously demonstrated that the ER calcium release channel, IP3R-1, is localized to the TBC-ER
MCS and that knocking it down inhibits the formation of the actin network cuffing TBCs. We have also
established that the ORP9 lipid exchange protein is localized to the TBC-ER MCS. Using an in vivo
knockdown approach to probe function, we injected the testes of Sprague-Dawley rats with ORP9
targeted siRNA and then examined the tissues morphologically with bright field, super-resolution (STED),
and electron microscopy. As determined with Western blots, we achieved a knockdown of ORP9 and
maintained the knockdown for 2-3 days with daily injections. Sections from ORP9 siRNA injected testes
had longer TBC tubes and fewer fused TBC bulbs compared to controls, and late spermatids were also
abnormally retained in the epithelium of knockdown tissue, indicating spermiation failure. Our results
suggest that ORP9 is necessary for establishing TBC length, and for normal bulb vesiculation and fusion,
most likely by changing the plasma membrane lipid profile of the TBC. These data also further support
the conclusion that TBCs are part of the normal mechanism of sperm release.

P1013

A nanoscopic reconstruction of the mammalian endocytic machinery
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Clathrin Mediated Endocytosis (CME) is one of the most trafficked endocytic pathways in a cell and
involved in a multitude of processes including cell signalling, nutrient uptake and membrane
homeostasis. More than 50 components are involved in this process, orchestrating the progressive
invaginating of the PM until a clathrin coated vesicle is released into the cytoplasm. The study of these
components by various visualization approaches have greatly advanced our mechanical
understanding of CME over the last decades, revealing an intricate and adaptive molecular machinery.
However, the structural arrangement of many endocytic proteins at the developing clathrin coat
remains unresolved. In part, this can be attributed to the low molecular specificity of high-resolution
imaging techniques including electron microscopy, and limited spatial resolution of conventional
fluorescence microscopy. Here, we propose a Single Molecule Localization Microscopy (SMLM) approach to resolve the molecular organization at distinct stages of mammalian CME. By optimizing this super-resolution microscopy method for multiple colours and three-dimensional imaging, we are able to visualize the developing clathrin coat while simultaneously capturing other endocytic components. In order to quantitatively analyse each snapshot of a CME event, we fit a spherical model to the SMLM data of a single site. This allows for a robust approximation of the endocytic progression per site and the generation of a spatiotemporal model of endocytic components. We provide a proof-of-principle by visualizing the assembly of AP2 in combination with clathrin, as well as the recruitment of dynamin2 to the neck of the budding vesicle. By expanding this analysis to additional endocytic protein pairs, we hope to contribute to a more complete picture of the nanoscale architecture of the CME machinery, and expand our understanding of the regulatory mechanisms involved in it.

P1014

Impact of AXL intracellular trafficking on its prometastatic activity in triple negative breast cancer
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Metastatic breast cancer, a form of the disease that has spread to secondary organs, is associated with poor prognosis. Triple-negative breast cancer is a subtype with a high propensity for metastasis and for which no targeted therapy is available. It is now established that expression of the receptor tyrosine kinase (RTK) AXL in multiple subtypes of breast cancer correlates with metastasis. More specifically, it has been shown that AXL contributes to sustain epithelial to mesenchymal transition (EMT) as well as focal adhesion dynamics in cancer cells which are two essential processes for cell migration and invasion that largely drive metastasis. Vesicular trafficking emerged as a crucial mechanism to control RTKs localization, diversify their signalling and promote their degradation. Deregulation of these processes can lead to RTK-dependent pro-oncogenic signalling. While trafficking of classically studied RTKs (e.g. EGFR or MET) is well-defined, how AXL traffics has not yet been studied. A collection of preliminary data has led us to hypothesis that AXL displays a unique trafficking route in TNBC cells that contributes to its pro-metastatic activity. Here, we took advantage of BioID to identify AXL’s proximity interactome which led us to identify potential AXL trafficking pathways. Among the candidates proximal to AXL, some are specifically involved in trafficking, such as RAB13, CAV1 or FLOT2. In order to characterize AXL trafficking pathways, we will perform classical endocytosis and recycling assays following depletion of the BioID candidates by siRNA. Building on a phosphoproteomic screen of AXL which identified proteins involved in trafficking, we will also test the potential contribution of AXL in the regulation of trafficking. By using AXL inhibitor, as well as phospho-mutants of AXL’s trafficking targets, we will determine the exact role of AXL-dependent phosphorylation in trafficking regulation and their impact on metastasis. In summary, this is the first study to comprehensively explore the impact of AXL trafficking in shaping the metastatic response in breast cancer. This could pave the way of identifying new targets that contribute to invasion that may be effective in hindering metastasis in TNBC, a cancer subtype with limited treatment options.
Weakly Internalized Receptors use coated vesicle heterogeneity to evade competition during endocytosis

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Uptake of receptors by clathrin-mediated endocytosis is a fundamental process that underlies signal transduction, nutrient import, and recycling of transmembrane proteins and lipids. In the complex, crowded environment of the plasma membrane, receptors are internalized when they bind to components of the clathrin coat, such as the major adaptor protein, AP2. Receptors with higher affinity for AP2 are known to be more strongly internalized compared to receptors with lower affinity. However, it remains unclear how receptors with different affinities compete for space within crowded endocytic structures. To address this question, we constructed receptors with varying affinities for AP2 and allowed them to compete against one another during internalization. As expected, internalization of a receptor with high affinity for AP2 was reduced when it was co-expressed with a competing receptor of similar affinity. However, receptors of low affinity for AP2 were surprisingly difficult to displace from endocytic structures, even when expressed alongside receptors with much higher affinity. How are these low affinity receptors protected from competition? Clathrin-coated structures that lack AP2 have recently been reported by several labs. When we examined structures with low AP2 content, we found that they were enriched in cargo of low affinity for AP2 and depleted of cargo with high affinity. These findings suggest that the heterogeneity of adaptor protein content across the population of endocytic structures enables internalization of diverse receptors. Given the critical role that internalization plays in receptor signaling, this effect may help to prevent strongly internalized receptors from interfering with the cell's ability to process signals from weakly internalized receptors.

Establishing and Maintaining Organelle Structure: ER and Lipid Droplets

Microtubule Glutamylation Specifies Polarized Distribution of Endoplasmic Reticulum

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The endoplasmic reticulum (ER) comprises functionally and morphologically distinct but interconnected structures including tubules, sheets, dense matrices and the nuclear envelope. While much progress has been made in understanding how the peripheral tubular network is formed, little is known about how the high-density perinuclear ER sheets and matrices are formed and distributed. We show that three ER membrane-bound proteins -- CLIMP63, p180 and kinectin -- preferentially bind centriolar, perinuclear and peripheral microtubules, respectively, to establish proper distribution and morphology of perinuclear ER. Mechanistically, p180 and kinectin selectively bind glutamylated and polyglutamylated microtubules, respectively. Knockout of these proteins or manipulation of microtubule populations or glutamylation status dramatically changes ER positioning, either more dispersed or more clustered, which also leads to correlative spatial rearrangements of almost all other membranous organelles. Our
Lipidomic changes impact the morphology of the endoplasmic reticulum.

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The endoplasmic reticulum (ER) is an important membranous organelle. Flat sheet-like cisternae and dynamic tubules define the complex network of the ER. While sheets are involved in synthesis, folding and secretion of proteins, tubules are recognised as the main sites of calcium storage, lipid synthesis, and ER’s contact with other organelles. Although the correct functionality of the ER is highly dependent on its structural diversity, the fine balance that regulates the ratio of ER sheets to tubules is not fully understood. In this work we demonstrate that the cellular lipid composition is crucial to maintain the ratio of ER sheets/tubules, suggesting that lipids are determinants of ER morphology. We conducted an siRNA screen in which HeLa cells were treated with 260 siRNAs targeting lipid biosynthetic enzymes. Removal of several of these enzymes, and the lipids they produce, and results in dramatic alternation of the relative amount of ER sheets and tubules. We have identified which lipids are changed in cells with morphological alterations and have analysed the impact of these lipidomic changes in functional assays. Our work is the first systematic analysis of how lipid composition influences ER structure and function.

**When is a tether not a tether? When it’s Ice2.**

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The study of membrane contact sites is at a relatively new stage where not all the components are well described. One proposed component in yeast is Ice2p, an integral endoplasmic reticulum (ER) membrane protein. Cells lacking Ice2p have less cortical ER, and the name "I-C-E" derives from lack of Inheritance of Cortical ER. Previous studies have indicated that a cytoplasmic loop in Ice2p reaches out to a partner organelle at membrane contact sites, with both lipid droplets and the plasma membrane being identified as partners. Using bioinformatics, we predict that Ice2p is homologous to SERINCs (serine incorporators), which are found universally in eukaryotes. The loop is not cytoplasmic, but is in the ER lumen, so Ice2p cannot be a tether. Since SERINCs are potent HIV restriction factors with no known molecular function, study of Ice2p together with the known yeast SERINC Tms1p, may reveal SERINC’s fundamental function, contributing to understanding of HIV restriction.

**Formation and roles of the specialized architecture of axonal tubular ER**

Continuity of ER through axons, dendrites and cell bodies potentially makes it a channel for long-distance communication, independent of action potentials or microtubule transport, like a “neuron within a neuron”. Axonal ER comprises a network of mainly smooth and tubular ER. Several proteins with intramembrane hairpin domains that model ER membranes, of the spastin, atlastin, REEP and reticulon families, are candidates to establish and maintain this network, since mutations affecting them cause an axon degenerative disease, hereditary spastic paraplegia (HSP). We aim to understand the mechanisms that maintain unbroken architecture of axonal ER over distances that are massive on a subcellular scale, and the functional roles of this architecture. We identified new markers for axonal ER in Drosophila, which show continuity through axons and synapses, judged by confocal and electron microscopy, and FRAP. EM also reveals an unusually small tubule diameter. Despite its apparent stability, the ER network in axons is highly dynamic. The amount and continuity of axon ER is controlled by ER-shaping HSP proteins including reticulons and REEPs. We hypothesize that (1) occasional ER gaps in HSP mutants could explain preferential degeneration of longer but not shorter axons; (2) additional unknown proteins are required to form axonal ER tubules; (3) homeostatic mechanisms maintain sufficient dynamic ER tubules in axons to avoid gaps in the network, and prevent accumulation of excess local ER; (4) continuity of ER membrane is important to its roles and in axon maintenance; (5) the minuscule lumen of axonal ER has functional consequences for ER continuity. To test these models, we are using forward and reverse genetic strategies to identify more of the machinery that forms the ER network. We find new proteins that localize to axonal ER. Generation of multiply mutant stocks lacking multiple intramembrane hairpin proteins suggests significant redundancy in the roles of axonal ER shaping proteins. We have also developed approaches to monitor Ca²⁺ in different organelle compartments, ER-mitochondrial contacts, and lumen diffusion, in larval motor neurons. These tools are starting to reveal physiological changes in presynaptic terminals with altered ER architecture, and now allow us to explore the physiological significance of the specialized architecture of axonal ER.

The Function of Hereditary Spastic Paraplegia-Associated Protein Spastin in Endoplasmic Reticulum Morphogenesis is Dependent on Microtubule Binding
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The endoplasmic reticulum (ER) is the largest organelle in the cell and performs numerous critical cellular functions. The ER is comprised of an interconnected membrane network of tubules and sheet-like membranes, extending from the nuclear envelope to the outermost cellular periphery. Emerging research has identified diseases linked to mutations in ER-shaping proteins, including hereditary spastic paraplegias (HSPs), as well as abnormalities in ER membrane contact sites in various neurodegenerative disorders. Hereditary spastic paraplegias (HSPs) are a genetically diverse group of inherited neurodegenerative disorders prominently affecting the longest corticospinal motor neurons. The disease is characterized by progressive spasticity of the lower limbs, for which there are no curative or preventative treatments. The most common forms of HSPs are due to autosomal dominant mutations in
one of three tubular ER-shaping proteins: spastin (SPAST), atalstin-1, and REEP1, with mutations in the SPAST gene accounting for the highest percentage (40-50%) of HSPs. Spastin is a microtubule-binding and -severing AAA ATPase enzyme expressed as two major isoforms from a single mRNA transcript via alternative translation. Although the two isoforms have different subcellular localizations, the overwhelming majority of mutations in SPAST are loss-of-function and will affect the expression of both isoforms. The larger M1 isoform is localized to the ER, while the shorter M87 isoform is predominately cytosolic; however, their individual contributions to disease pathogenesis is unknown and the function of M1 spastin as a tubular ER shaping protein remains obscure. We use CRISPR/Cas9 gene editing to generate isoform-specific knockout cell lines and found that deletion of M1 spastin results in loss of reticular ER and expansion of sheet-like ER to the cellular periphery, while normal ER morphologies persist in cells specifically deleted for M87. Furthermore, we identify the protein domains that are necessary for tubular ER shaping by spastin, which requires both the hydrophobic hairpin region conferring ER localization and the microtubule-binding domain, but microtubule severing activity is not required. Additionally, in cells deleted for all spastin isoforms, only M1 containing an intact microtubule binding domain could rescue the reticular ER network. Furthermore, live cell single-molecule imaging approaches were used to examine the specific dynamic nature of spastin and its interaction with microtubules. Understanding the functional contributions of spastin isoforms will be crucial for understanding the etiology of HSPs and for finding new therapeutic targets.

P1021

Cryo-EM structure of yeast seipin reveals critical functional elements

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Seipin, an oligomeric transmembrane protein of the ER, is essential for the formation of normal cytoplasmic lipid droplets (LDs) and mediates LD contact with the ER. Here we report the molecular structure of seipin (Sei1/Fld1) from Saccharomyces cerevisiae. The cryo-EM map of yeast seipin shows a homo-decamer with a length of 147 Å, height of 33 Å, and a central pore of 26 Å diameter. Each seipin subunit has an ER luminal, β-sandwich domain conserved in human and Drosophila seipin with similarities to domains of lipid-binding proteins such as NPC2. Our structure reveals key residues mediating the monomer-monomer interactions and the luminal juxta-membrane (linker) region (see also Arlt, Folger, et al, this conference). A point mutation in the monomer-monomer interface abolished oligomerization of isolated luminal domains, but not of full-length seipin. Co-evolving pairs of amino acids between the two transmembrane (TM) segments suggest that inter-TM domain interactions provide additional contacts for oligomer formation. Consistent with this model, seipin variants with point mutations in these residues are poorly expressed suggesting instability and have very little seipin function even when overexpressed. The yeast seipin structure provides a more complete understanding of the seipin oligomeric architecture and identifies important elements tying seipin structure to function.
Roles of the unfolded protein response in the mammalian cell cycle
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During the cell cycle, genome duplication is coupled to the multiplication and growth of organelles, which requires membrane biosynthesis. Since membrane biosynthesis starts in the endoplasmic reticulum (ER), ER growth would be a pre-requisite for cell division. Because the unfolded protein response (UPR)—the central mechanism that maintains ER integrity—increases the size and protein-processing capacity of the ER, we reasoned that it may oversee ER physiology during the cell cycle. To investigate ER growth and activation of the UPR during the cell cycle, we used fluorescent reporters of cell cycle progression that allow us to separate G1 and G2 cell populations. Our data show that mammalian cells increased in size and granularity during interphase. These hallmarks were correlated with an increase in ER-resident protein content, suggesting that the ER enlarges in preparation for cell division. Moreover, we observed that the mRNA levels and activity of the kinase/RNase IRE1, the most conserved UPR sensor/transducer, were higher in G1 as compared to G2, hinting at a role for IRE1 in the G1/S transition. Consistent with this observation, we found that inhibition of IRE1 delayed progression through the G1/S boundary. In addition, we have found that IRE1 activity is suppressed by PKMYT1, an ER- and Golgi apparatus-associated G2/M cell cycle checkpoint kinase, suggesting that PKMYT1 exerts regulatory control over IRE1 prior to cell division. Taken together, our results show that mammalian cells engage a physiological UPR involving IRE1 signaling during cell cycle progression.

Dissecting seipin function from cryo-EM structure: Identification of key residues in the luminal inter-subunit interface, juxtamembrane region, and transmembrane domains
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The loss of function of seipin results in severe lipodystrophy in humans, in which adipose tissue fails to develop. Seipin, an ER integral membrane protein, is responsible for the generation of functional lipid droplets in virtually all eukaryotic cells. The core seipin structure in *S. cerevisiae* is a homo-decamer with radial symmetry, as determined by cryo-EM (see Arlt, Sui, et al, this conference). Mutations in the genome were generated in seipin residues predicted to line the inter-subunit luminal interface, the luminal juxtamembrane region, and the two transmembrane domains (TMDs). The effects on lipid droplet number and size were assessed through an automated pipeline. Effects of mutations were usually enhanced in a perilipin (Pln1)-deficient background, confirming the interplay of these two proteins. Single mutations in R178 and W186, two residues which may interact across the inter-subunit
cleft had the strongest effect on LD number and size. Seipin-R178A had low steady-state levels, which was brought to normal with overexpression of Ldb16, a seipin-interacting protein. However, the R178A LD phenotype was not corrected by overexpression of R178A or Ldb16. The mutation E172A partially compensated for the R178A mutation, suggesting the two have opposite roles in inter-subunit interactions. Shortening or scrambling residues in the juxtamembrane region, a domain that is presumed flexible as it is not visualized in the cryo-EM structure, led to heterogeneous droplets and low steady-state seipin levels. Finally, mutations in conserved amino acids that may crosslink the two TMDs has strong LD phenotypes and low expression, and their overexpression did not improve phenotype. Overall, our seipin mutants highlight important structural elements for seipin assembly and function, and they may be useful tools for further dissection of the role of this protein in lipid droplet biogenesis and maintenance.

G1, G1-S, and S Phase Regulation: Cell Size and Cell Fate

P1024

Modulation of single cell volume and mass growth by intrinsic and extrinsic factors
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Cells that make up the same tissue or organ tend to have a stereotypic physical size, while variability in cell size is a marker of abnormal cell growth. A well-defined cell size with low variance is crucial for the intimate coordination between cell growth and division, which drives physiological functions like cell proliferation, differentiation, and embryonic development. But, how size homeostasis is achieved and what size parameter is measured is still an open question for mammalian cells. In this work, we combine two independent methods for single live cell size measurement: quantitative phase microscopy to measure cell dry mass and fluorescence exclusion, to measure cell volume, on a number of cell types over a complete cell cycle. Specifically, we aim to: 1) Understand fluctuations in mass and volume growth during cell-cycle and, 2) Study mechanisms that couple mass and volume thus modulating cell density. We show that cells grow linearly and steadily in mass without many plateaus, unlike volume, over the complete cell cycle. Additionally, mass added across each cell cycle is independent of the initial mass of the cell, corresponding to the same adder-like behaviour observed for volume (Cadart et al Nat Com 2018). We also show that the density of cells is not constant, and instead the cells are born denser as mass increase starts immediately at mitotic exit whereas volume increase is delayed in new born cells (Zlotek-Zlotkiewicz, JCB 2015). Our initial results show that density starts to decrease several hours into the cell cycle and is proportional to mean mass growth rate rather than volume. Density perturbations are also seen during cell spreading on adhesive substrates. We aim to understand the factors which modulate the density and mass/volume coupling which might also correspond to dynamic cell shape changes due to migration/spreading in adhesive cultured cells. This work aims at a fundamental understanding of how cells grow and regulate their size. It will also elucidate functional consequences of modifications in mechanisms of mass/volume coupling on cellular functions. References: 1. Cadart, C., Monnier, S., Grilli, J., Sáez, P. J., Srivastava, N., Attia, R., … Piel, M. (2018). Size control in mammalian cells involves modulation of both growth rate and cell cycle duration. Nature Communications, 9(1), 3275. https://doi.org/10.1038/s41467-018-05393-0 2. Zlotek-Zlotkiewicz, E., Monnier, S., Cappello, G., Le Berre, M., & Piel, M. (2015). Optical volume and mass measurements show that mammalian cells
P1025

Size homeostasis in proliferating mammalian cells

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In proliferating cells, variability in cell size can arise from variability in growth rate, cell cycle length, and the ratio between the two progeny cells. During recent years, more evidence support that mammalian cells control their targeted size mainly by adjusting the length of the G1 phase. However, the size control in G1 is insufficient to guarantee the desired size variability at the end of the cell cycle. Indeed, by using the computationally enhanced Quantitative Phase Microscopy (ceQPM), we discovered the size variability of adherent mammalian cells is modulated throughout the cell cycle. The cells adjust the length of multiple cell cycle phases and also regulate the growth rate to maintain the size homeostasis. When the primary adjusting mechanism fails, other mechanisms take over to compensate for the effect. We found the size variability is robustly maintained even if the targeted size has been changed by drug treatments or environmental conditions. More interestingly, we found various forms of growth regulation utilized by different cell lines, suggesting the multiplicity and flexibility of growth control. We plan to use several quantitative single-cell methods to investigate the molecular mechanisms of the growth regulations. We hope to share some of the results at the conference.

P1026

“When bigger is not better”: Whole genome duplication generates DNA damage by compromising DNA replication dynamics.

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Polyploidy results from the gain of complete chromosome sets. Polyploidization can be developmentally programmed to sustain cell and tissue functions. Conversely, when non-programmed, polyploidization generates high levels of genetic instability. Although elimination pathways are described, a growing amount of evidence shows that non-programmed polyploid cells can escape these pathways and contribute to pathological conditions, including cancers. Indeed, about 40% of human tumors have experienced whole genome duplications (WGD), which appear to fuel highly abnormal karyotypes favoring tumor evolution. However, the molecular mechanisms linking WGD to genetic instability remain poorly understood. To investigate this question, we combined in vivo approach in Drosophila melanogaster with mechanistic approach in the human non-tumoral RPE-1 cell line. We first developed several strategies to generate mono- or multinucleated polyploid cells. Independently of the strategy used and of the number of nuclei, we observed DNA damage generation, revealed by γH2AX foci, within the first interphase upon polyploidization. Interestingly, γH2AX signal covered large part of the nucleus, suggesting that DNA damage is not generated at a specific region of the genome. By synchronizing polyploid cells in G1, we fully prevented DNA damage, whereas cells released in S-phase accumulated γH2AX foci and replication stress markers. More importantly, inhibition of DNA replication fully prevented DNA damage in polyploid cells. Previous data suggested that DNA damage in polyploid cells
resulted from errors during mitosis leading to cell cycle arrest in the next G1. Here, we show that even before mitosis, polyploid cells experience high levels of DNA damage, causing by the first DNA replication. These observations are crucial since such genetic instability could promote the acquisition of mutations allowing the proliferation of unstable polyploid clones. Now, by characterizing DNA replication dynamics using 4D quantitative live imaging, we show that full activation of replication sites is delayed in polyploid cells. Moreover, we screen for DNA replication factors and identified a group of proteins, including MCM2, that does not scale up with DNA amount in polyploid cells, suggesting that some replication factors could be limiting generating then DNA damage. This model implies that physiological polyploid cells, that do not experience genetic instability, are able to scale up factors essential to prevent DNA damage. In support of this model, we observe that some proteins that maintain genetic stability are more expressed in physiological polyploid tissues in vivo in Drosophila brain compare to our model of non-programmed polyploidy.

P1027

Evidence for a novel feedback loop in cell size control in budding yeast

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Size control in cells is critical in preventing pathology and retaining proper function. Cell size homeostasis results from the coordination between cell growth and division. In budding yeast, Start marks the cells’ commitment to division. The G1 cyclin, Cln3, binds to the cyclin-dependent kinase, Cdc28, leading to inhibition and nuclear export of the transcriptional repressor, Whi5. This de-represses the transcriptional activator complex, SBF (Swi4-Swi6), which activates expression of the ~200 genes of the G1/S regulon. Two other G1 cyclins, Cln1 and Cln2, are among the genes in this regulon, setting up a feedback loop to render Start irreversible. The dynamics of this feedback loop and the expression of these G1-cyclins is not well-characterized. Here, for the first time, using 2-photon scanning Number and Brightness microscopy, we examined the expression dynamics of GFPmut3 fusions of the G1-cyclins in individual cells. In wild-type cells, we observed sporadic peaks of nuclear Cln3 at 1 and/or 2 hours before budding, while Cln1/2 expression peaked at or just after budding. We recently reported that the steep build-up of the DNA binding subunit, Swi4, of SBF, is a direct, dose dependent determinant of Start. Here we show that the size/time dependent build-up of Swi4 is 50% lower in Cln3 deletion cells, suggesting positive feedback of Swi4 on its own production. Supporting this hypothesis, we found that Swi4 build-up was 50% lower in a strain deleted for the SBF and MBF target sites in the Swi4 promoter, as well as for a strain containing conditionally suppressible Cdc28 kinase allele treated with an inhibitor drug. These observations are interpreted in the framework of a model for cell size control.

P1028

G1 - S regulators coordinate cell cycle progression and cell fate of cyst stem cells in the drosophila testis

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Stem cells maintain tissue homeostasis by balancing self-renewal and differentiation. Fully differentiated cells exit the cell cycle, so we asked whether disruption of the cell cycle influenced cell fate. We use the
Drosophila testis to study this coordination. The testis niche supports two stem cell populations, germ line stem cells and somatic cyst stem cells (CySCs). CySC proliferate while their differentiating daughter cells do not, providing an ideal model to study how fate and cell cycle are coordinated. Here, we focus on regulation of G1/S transition and its effect on CySC fate. We show that blocking the G1/S transition causes premature differentiation, whereas knocking down Rbf, the homologue of Retinoblastoma that inhibits the G1/S transition, expanded the CySC population and blocked its differentiation. Rbf functions by inhibiting a transcription factor complex, E2f1/Dp. Surprisingly, E2f1/Dp activity was not required for self-renewal or cell cycle progression, suggesting that the endogenous role E2f1/Dp activity is not in promoting cell cycle progression but to prevent differentiation in cycling cells. To determine how Rbf inhibited differentiation, we analysed gene expression and identified changes in genes regulating mitochondrial biology and energy production. Promoting mitochondrial biogenesis and function by co-expressing the PGC1α homologue, Spargel (Srl) and Delg/NRF-2 in Rbf knockdowns rescued differentiation of CySCs but also led to ectopic cycling in differentiated cells. Thus, our results indicate that Rbf coordinates cell cycle exit with differentiation by inhibiting E2f1/Dp and promoting a permissive metabolic state.

Gene Structure and Transcription

P1029

Compartmentalized RNA transcription in human cells

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The spatial organization of actively transcribed genes and their cognate nascent transcripts in the nucleus of mammalian cells remains incompletely understood. Using confocal microscopy, we visualized a tandem array of ~335 recombinant human β-globin gene copies, its associated RNA polymerase II (Pol II) and nascent RNA in human cells. We imaged the genome-integrated gene array using a targeting guide RNA and catalytically dead CRISPR-associated system 9 (dCas9) fused to the green fluorescent protein (GFP). We detected Pol II by immunofluorescence. Intronic RNA was labelled in live cells by tagging with MS2 stem loops and MS2 coat protein fused to fluorescent proteins. Fluorescence in situ hybridization was used to localize exonic sequences. Three-dimensional reconstructions of z-stack micrographs were analyzed. We observed a partial co-localization between Pol II and the gene array locus, suggesting that not all gene copies were simultaneously transcribed. Partial co-localization was also observed between introns and exons of nascent transcripts and Pol II, suggesting that polymerases and newly synthesized RNAs are organized as distinct compartments in the nucleus. Our findings contribute novel insights into structural aspects of gene expression. This work was supported by FCT - Fundação para a Ciência e a Tecnologia, Portugal, through the postdoctoral fellowship SFRH/BPD/102323/2014 to J.P.

P1030

Study on the DNA adduct and gene mutations caused by chemical composition of aerosol in vaping

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Vaping, or inhaling of e-cigarette vapor, may have long term health consequences. Vaping may initiate and promote carcinogenesis, leading to lung cancer. However, there is a lack of prospective epidemiological studies that provide evidence on how exposure to vaping may lead to lung cancer. This research used biochemical and computational simulations to examine how vaping affects the metabolic and stereochemical conversion of benzo[a]pyrene to enzo[a]pyrene-7,8-dihydropyrene-9,10-epoxide, a chemical carcinogen identified in electronic cigarettes. The progression of carcinogenesis occurs during the enzymatic metabolism from benzo[a]pyrene to benzo[a]pyrene-7,8-dihydropyrene-9,10-epoxide (BPDE). During this process, this paper showed how the intercalation resulted in the formation of guanine benzopyrene through binding with guanine bases in the DNA. Computational chemistry and Density Functional Theory (DFT) with a molecular editing program were further used to find the compounds’ biophysical properties such as active energy and optimized molecular shape in assessing the thermodynamic stability of the molecular genes. Results showed that the optimization energy of the studied molecule increased as the weight and size of molecules increased. The difference between the optimization energy of G:C and G-BPDE:C (363.227 kJ/mol) was similar to that of the difference of the optimization energy between G:C, C:G and G-BPDE:C, C:G (353.408 kJ/mol), meaning that BPDE will add about 358.318 kJ/mol to the optimization energy of the G:C and G-BPDE:C complex. In all four optimizations, the hydrogen bonds were not accounted for by the program, and were ultimately broken during the process of optimizing the energies. Optimization energy of the complex G-BPDE:C and C:G (3093.631 kJ/mol) showed highest among the tested samples.

P1031

Extracting Transcriptional Bursting Parameters from Single Nascent RNA Microscopy Data

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Gene expression is a dynamic and stochastic process, resulting in heterogeneity within and between cells, which can affect essential cell fate decisions of an isogenic population. Countless research efforts have been made to pinpoint the origin and consequences of such heterogeneity, but a mechanistic understanding is lacking. A simple yet experimentally validated model accounts for the production of RNA in bursts, which can theoretically be described by the existence of on/off states for a gene and the stochastic transcription during the on states. Yet recent data suggest that gene regulation is more complex, characterized by more states and regulatory steps, e.g., for two neighboring genes. Although single molecule FISH (smFISH) permits detecting single nascent RNAs at the transcription site with high spatial resolution, such an approach is performed on fixed cells and, therefore, cannot access the temporal switching between on/off states. Live-cell imaging technologies based on MS2/PP7 stem-loops access the temporal dimension by measuring the number of nascent RNAs over time. Here we present a novel analysis strategy that maximizes the spatio-temporal resolution by combining dual-color single molecule FISH (smFISH) and dual-color live cell imaging of nascent RNAs data of two galactose-responsive neighboring genes in budding yeast, i.e., GAL1 and GAL10. A model of transcription co-regulation in which GAL1 and GAL10 show correlated bursts is proposed. The simultaneous fitting of smFISH and live-cell nascent RNA distributions through stochastic simulations allows us to extract kinetic parameters of transcription, such as the burst sizes and frequencies for both GAL genes. We also determined that GAL1 and GAL10 show highly correlated bursts, with ~70% of bursts from GAL10 co-occurring with bursts from GAL1.
Mammalian PAF49, the ortholog of the nonessential yeast RNA Polymerase I subunit Rpa34, is essential for cell proliferation

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The regulation of ribosome biogenesis (RB) plays a central role in maintaining cellular homeostasis and supporting cell growth. The rate-limiting step in this process is transcription of the ribosomal RNA genes by RNA polymerase I (Pol I). Dysregulation of RB can contribute to pathologies such as cancer, cardiac hypertrophy, and ribosomopathies. Further, many chemotherapeutic drugs inhibit either rDNA transcription or rRNA processing, but have many off-target effects that limit their usefulness. Many pathways play a role in the regulation of rDNA transcription. Two mammalian factors that are involved in this regulation are Polymerase Associated Factor (PAF53) and PAF49. The purpose of this study is to determine the role(s) the PAFs play in regulating rDNA transcription and characterize the downstream physiological effects of directly inhibiting their functions. Previously, our lab used CRISPR/Cas9 in conjunction with an auxin inducible degron to target and rapidly knock down PAF53. We determined that PAF53 is required for rDNA transcription and cell proliferation. Further, we identified a novel DNA-binding domain in PAF53. A putative helix-turn-helix motif within this domain was found to be responsible for its DNA-binding activity. Disruption of this helix-turn-helix motif in full length PAF53 causes the protein to be nonfunctional in vivo. Recently, we have used this same system to perform mirror studies in HEK293 cells that have been engineered to inducibly knock down PAF49. Our preliminary data show that PAF49 is also required for both rDNA transcription and cell proliferation. These results contradict the studies performed in yeast that demonstrate that the yeast ortholog Rpa34 is a nonessential protein. Further, our lab has found that PAF53 and PAF49 are co-regulated posttranslationally. When PAF49 is knocked down, PAF53 is also degraded and vice versa. These findings are significant because they aid in further understanding the process of rDNA transcription by Pol I and the physiological consequences of inhibiting this process, i.e. nucleolar stress and cell arrest and/or death. The results from this study have also lead to the discovery of novel drug targets that could be utilized in effective cancer therapies.

BbRad51 roles in babesia bovis survival


Babesia bovis is a tick-borne hemoparasite of cattle, and a microaerophile. In order to optimize its growth environment and avoid splenic passage, B. bovis-infected erythrocytes cytoadhere to the capillary and post-capillary venous endothelium. Cytoadhesion is achieved through the highly variant, heterodimeric VESA1 ligand expressed on the infected erythrocyte surface. VESA1a and 1b subunits undergo rapid antigenic variation to maintain their ability to cytoadhere despite an ongoing host immune response capable of abrogating cytoadhesion. Members of the large ves multigene family encoding the VESA1 subunits, vary through in situ transcriptional switching (isTS) and segmental gene conversion (SGC). SGC has long been assumed to be a product of homologous recombination (HR), a
form of DNA repair. HR is considered to be dependent upon the protein, Rad51, which stabilizes long 3’ overhangs at the ends of double-strand DNA breaks and mediates invasion of an unbroken allele or paralog by the broken end. **Objective:** Our objective was to assess the essentiality of BbRad51 to SGC during *B. bovis* antigenic variation. **Methods:** To achieve this we knocked out the *Bbrad51* gene by homologous recombination and assessed for phenotype. **Results:** *Bbrad51* knockout resulted in a phenotype of elevated sensitivity to methylmethane sulfonate (MMS) but not to gamma-irradiation, and a lack of effect on growth. Sensitivity to MMS could be complemented by reinsertion of a second *Bbrad51* locus on the artificial chromosome, BbACc3. Remarkably, *Bbrad51* knockout failed to abrogate SGC. Rather, SGC continued at rates comparable to wild-type at any transcriptionally active ves locus, but isTS was slowed significantly, suggesting an interplay of BbRad51 and epigenetic modifier components. Moreover, the distribution of SGC tract sizes was skewed toward slightly, but statistically significant, shorter lengths. The net result was an overall reduction in the variety of ves mutations generated and presumably of VESA1 mutant isoforms. **Conclusions:** BbRad51 is not essential to SGC, but influences ves gene regulation. We anticipate that its loss would result in a reduced capacity to evade host immune responses and would render the parasite compromised in its ability to establish persistent infections.

P1034

Understanding the role of the SAGA complex in gene expression heterogeneity and transcriptional memory

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Regulation of gene expression is essential for transcription of genes that initiate long-term changes in cell fate. Despite many organisms having robust mechanisms to control gene expression dynamics there still exists fluctuations in the number of RNA and proteins expressed in genetically identical cells. This gene expression heterogeneity may be utilized during development and serve as a non-genetic mechanism to encode phenotypic diversity. One potential source of this heterogeneity is differences in gene expression due to cells capability to have memory of past events or cell states. Recent work has indicated that in response to a stimulus transcriptional memory can occur from changes in histone modifications that can influence how cells respond to future stimulus exposures. One multiprotein complex that plays an important role in determining expression heterogeneity is the transcriptional co-activator Spt-Ada-Gcn5-acetyltransferase (SAGA). Two subunits of the SAGA, histone acetyltransferase Gcn5 and ubiquitin protease Ubp8, can induce histone modifications and affect the recruitment of gene regulatory factors. The question then remains of how and to what extent SAGA affects transcriptional memory and noise in gene expression to enable cells to most efficiently respond to signals in fluctuating environments. An ideal system to study how SAGA affects memory and heterogeneity in gene expression is the inducible osmotic stress response system in budding yeast. In *S. cerevisiae* upon hyperosmotic shock, the high-osmolarity glycerol (HOG) pathway induces nuclear localization of Mitogen Activated Protein Kinase (MAPK) Hog1 which recruit’s chromatin-modifying complexes including SAGA to protect cells from future osmotic stress. **We hypothesis that Gcn5 and Ubp8 differentially regulate production and cytosolic availability of mRNA to control heterogeneity and memory in stress response gene expression.** To determine how Gcn5 and Ubp8 affect single cell gene expression, we will conditionally deplete them and measure changes in expression of osmotic stress response reporter genes. These experiments provide the basis for building probabilistic kinetic models.
that can interrogate how regulatory mechanisms give rise to cell-to-cell differences in gene expression, and quantitatively determine how Gcn5 and Ubp8 affect initiation, elongation, and export of RNA upon stress. Preliminary model simulations altering gene regulatory reaction rates predict that changing rates of chromatin state transitions, initiation, and export have distinct outcomes on gene expression heterogeneity. These experiments will help us to better understand how SAGA coordinates gene expression dynamics to ensure that cells can adapt to stress and survive in fluctuating environments.

P1035

Identification and Annotation of Genetic Sequences in Drosophila ananassae, contig17
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The genome of Drosophila melanogaster has been a highly studied genome in biology for the past twenty years. D. melanogaster is a model organism for studying the developmental and cellular processes common in other eukaryotes. Here, the D. melanogaster genome was used as a reference for identifying genes and genomic elements in contig 17, an approximately 18,000 bp region of the related Drosophila ananassae species. The analysis of the sequences and data collection was done using open source computational genomic tools for sequencing, gene-prediction, and genome browsing. The resources used during this project were obtained through the Genomic Education Partnership (GEP). GEP is a bioinformatics program sponsored by Washington University, in St. Louis. The analysis of contig17 of the Drosophila ananassae genome yielded the presence of four genes, homologous with genes Ddx1, Rich, CG11523, and JMJD7 of D. melanogaster. No incomplete genes or nonconsensus regions were found present within this contig.

P1036

Lodestar, a protein with a dual role in mitotic transcription inactivation and sister chromatid resolution
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Mitosis is essential for life. This process requires drastic changes in chromosome structure that parallels with a major switch off in transcriptional activity. While it has long been assumed that increased compaction would render mitotic chromatin inaccessible for transcription, the interplay between mitotic chromosome organization and transcription termination remains elusive. While looking for novel genetic interactions with chromosome assembly factors, we uncovered a poorly characterized helicase-like protein – TTF2 (transcription termination factor 2) / Lds (lodestar in Drosophila melanogaster). We found that Lds is predominantly cytoplasmic during interphase and localizes to mitotic chromosomes a few seconds prior to Nuclear Envelope Breakdown, suggesting it may be critical for mitosis-specific chromatin events. Accordingly, absence of Lds results in severe segregation errors (1), mostly characterized by chromatin bridges during anaphase. However, how Lds ensures faithful chromosome segregation remains unknown. Given that absence of Lds results in severe chromatin bridges, we hypothesize that it aids in the process of sister chromatid resolution. Accordingly, we found that Lds interacts with the major enzyme that resolves sister chromatid intertwines - Topoisomerase II – and localizes to chromatin bridges at mitotic exit. These findings suggest Lds cooperates with Topoisomerase II to facilitate sister chromatid resolution. Strikingly, previous studies support that Lds holds transcription
termination activity *in vitro* (2), which would indicate that the observed segregation defects could be (at least partially) attributed to impairment in transcription termination. Indeed, we show that Lds is required for timely release of transcripts from chromatin at mitotic entry. Using a system to monitor ongoing transcription in real time (3) we see that in embryos with reduced levels of Lds retain transcripts at mitotic chromatin, despite normal mitotic progression and chromosome compaction. We propose that Lds has a dual role during mitosis, by ensuring prompt inactivation of transcription and cooperating in sister chromatid resolution. Ongoing work is aiming to dissect how these two functions are mechanistically linked and the contribution of these two processes for mitotic fidelity.


**P1037**

**Retracing the steps of early oncogene activation**  

DNA replication depends on a highly organized series of steps to ensure complete genome duplication in S phase. In addition, molecular events in the previous G1 phase (origin licensing) are critical for DNA replication preparation. Origin licensing involves loading of the DNA replication helicase, the minichromosome maintenance complex (MCM), onto origins of replication. Origin licensing is essential for pre-establishing conditions for efficient DNA replication. Origins are then fired/activated at the beginning of S phase. Insufficient licensing in G1 (“underlicensing”), results in incomplete DNA replication. Governing this process are the G1/S cyclins, “essential workers” that ensure orderly progression through the cell cycle. If G1/S cyclins become altered/mutated, they can become oncogenic leading to replication stress and genomic instability, a hallmark of tumorigenesis. This is relevant because some cancers have elevated cyclin E and elevated cyclin E impairs origin licensing. To study this, we generated inducible cell lines that overexpress each G1/S cyclin, optimized an analytical flow cytometry assay to study underlicensing in single cells, and studied proliferation. Overproducing cyclins D and A did little to affect proliferation. In stark contrast however, overproducing cyclin E, whose activity briefly peaks at the G1-S transition, truncated G1 length, leaving less time for cells to license their origins and induced subsequent underlicensing. Furthermore, cells that chronically overproduced cyclin E entered a phase of significantly reduced proliferation relative to control cells. Over time, these cells managed to adapt and proliferate faster while still chronically overproducing cyclin E. During their descent into proliferation stalling these cells incur DNA damage and are hypersensitive to replication inhibitors but then adapt and proliferate faster than control cells. Reducing cyclin E to normal levels in the adapted cells restored normal licensing, but this normalization required a few weeks. These results suggest that we have recapitulated (1) early effects of oncogene activation, (2) how cells avoid oncogene-induced senescence, and (3) how cells adapt to high levels of cyclins. In-depth analysis of these adapted cells will help our understanding of tumorigenesis and may reveal unique vulnerabilities in cyclin-overproducing cancers.
Growth Factor Signaling

P1038

Epidermal Growth Factor Receptor signaling requires specialized clathrin-labeled structures and necessary accessory proteins

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The Epidermal Growth Factor (EGF) Receptor (EGFR) controls many aspects of cell physiology, including growth, survival, migration and metabolism. Importantly, upregulation of EGFR contributes to proliferation and survival of many cancers. Upon binding its ligand, EGFR activates several signaling intermediates, and simultaneously is recruited to clathrin-labeled structures (CLSs). We previously uncovered that perturbation of clathrin, but not of receptor endocytosis, impairs EGF-stimulated activation of Akt signaling. Also, some EGFR signaling intermediates such as phosphorylated Gab1 are enriched with a subset of CLSs. We have also uncovered that the clathrin-binding protein target of myb-like 1 (TOM1L1) and the TOM1L1-binding Src-family kinase Fyn are recruited and enriched in a subset of CLSs. We proposed that CLSs have a direct role controlling EGFR signaling at the plasma membrane prior to receptor internalization, and the clathrin-accessory proteins Fyn and TOM1L1 are necessary in CLSs signaling. How CLSs and clathrin control EGFR signaling is not well understood, which we have examined here. We uncovered that the perturbations of TOM1L1 or Fyn phenocopy perturbations of clathrin heavy chain with regards to EGFR signaling. We generated stable cell lines for inducible controlled expression of various mutants of TOM1L1 and Fyn at near-endogenous levels to perform knockdown-rescue experiments to dissect the molecular mechanism by which TOM1L1 and Fyn contribute to EGFR signaling. Using total internal reflection fluorescence microscopy of cells expressing fluorescent clathrin, TOM1L1 and/or Fyn at near-endogenous levels, together with automated image analysis, we find that Fyn and TOM1L1 are selectively recruited to a subset of CLSs. CLSs that recruit TOM1L1 and Fyn exhibit unique properties, such as distinct lifetimes and Epsin recruitment. Silencing of TOM1L1 or Fyn impact dynamics of CLS harboring EGFR. These results suggest that TOM1L1 and Fyn are recruited to a distinct subpopulation of signaling-specialized CLSs that mediate certain aspects of receptor signaling directly at the plasma membrane.

P1039

Identification of galectin-7 as a new link between EGFR and E-cadherin

EGF is known to be a key player in cell differentiation, proliferation, migration and epithelial homeostasis. It mainly acts through ligation to its receptor EGFR which in turn triggers its phosphorylation and downstream-signaling pathways. EGFR activity is also regulated by its glycosylation state which influences its binding capacities. These glycosylations can be recognized by several molecules such as galectins, proteins belonging to a family of soluble lectins implicated in multiple processes. They are characterized by the presence of an evolutionary conserved carbohydrate recognition domain (CRD) and an affinity for β-galactosides containing sugars. Galectin-7, a soluble lectin, is expressed in pluristratified epithelia such as the skin and has been shown to be involved in cell differentiation. Thus, we observed a thickening of epidermis in mice deficient for galectin-7. Indeed, in this work we demonstrate that galectin-7 via its CRD regulates EGFR function by a direct interaction with its extracellular domain hence modifying its downstream signaling and endocytic pathway. This regulation notably impacts cell proliferation therefore underlying the phenotype observed in mice. We had previously pointed out that galectin-7 also directly binds E-cadherin independently of this CRD and regulates its plasma membrane dynamics. Interestingly, we now reveal that galectin-7 bridges E-cadherin and EGFR together and propose an in silico model. This study highlights a novel molecular interaction between EGFR and E-cadherin, two major regulators of the balance between proliferation and differentiation.

P1040

Role of FOXO transcription factor dynamics in determining cell fates following EGFR inhibition

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Epidermal Growth Factor Receptor (EGFR) tyrosine kinase inhibitors (TKIs) are a first line treatment for patients with advanced non-small cell lung cancer (NSCLC) who have specific hyperactive mutations in EGFR. Though patients have seen increases in survival after EGFR TKI treatment, resistance often develops. Indeed, EGFR inhibitor treatment induces both cell-cycle arrest (allowing for cell survival) and death in cancer cells. The FOXO family of transcription factors have been implicated in both responses following EGFR inhibition. However, it is unknown how the FOXO network ‘decides’ between these two oppositional programs to enact following EGFR inhibition. Protein dynamics, meaning how the levels or location of a protein changes over time, is a mechanism cells use to encode information about cellular fates. The p53 system is an established example of a dynamically regulated system—it responds to multiple inputs and is capable of enacting multiple outputs. FOXO’s network motif is similar to the p53 system making it a good candidate for dynamic regulation. We have tagged Foxo3a with a fluorescent marker in PC9 NSCLC cells to determine whether FOXO dynamics dictate cell fate following EGFR TKI treatment. Using live cell microscopy on this line, we have acquired FOXO nuclear cytoplasmic shuttling dynamics as well as cell fate (death/arrest) in single cells. Using time-series analyses we are trying to determine if there are dynamic patterns associated with each fate following EGFR inhibition. Additionally, FOXO nuclear cytoplasmic shuttling (pulsing) is seen at certain doses of EGFR inhibition, which suggests the presence of feedback in the PI3K/Akt/FOXO pathway. We have developed a drug screen using immunofluorescence to identify the sources of feedback in the system after EGFR inhibition as it hypothesized that the presence of feedback loops that reactivate pathway components after inhibition result in decreased treatment efficacy. Ultimately, through our studies, we hope to gain a better understanding of this non-genetic mechanism cells use to determine their fate. Our long-term goal is to identify dynamic patterns that push cancer cells to more terminal fates following treatment.
P1041

The Epidermal Growth Factor Receptor Dimer is Activated by Binding with a Single Ligand Molecule
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The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that serves as a master switch for signaling pathways that control cell proliferation, differentiation, migration and survival. Hence the receptor plays crucial roles in embryogenesis and organogenesis, and its impairment results in defects in various organs. EGFR is also among the most frequently altered proteins in human cancers such as lung cancer and colorectal cancer, and is one of major targets for cancer therapy since inappropriate activation of the EGFR network contributes to a number of processes in cancer development, progression and invasion. It has long been thought that ligand binding activates EGFR by inducing a dimeric structure of the receptor, in which intracellular kinase domains trans-autophosphorylate each other (the “ligand-induced dimerization model”). However, there are numerous studies indicating that EGFR exists as a preformed dimer prior to ligand binding. Consistent with this preformed dimeric structure, negative cooperativity has been observed when EGFR binds its ligand. It is vital to elucidate mechanisms underlying EGFR activation for understanding how mutations of the receptor cause cancers as well as for developing pharmaceuticals for cancer therapy. To observe the negative cooperativity in vivo, in the present study, we have observed the ligand-receptor interaction on the cell surface at the single molecule level. Using a three-color total internal reflectance fluorescence microscope (TIRFM), EGFR binding with EGF ligands separately labeled with two different fluorescent dyes was observed, together with the receptor binding to GFP-tagged Shc1, a downstream effector protein that binds to phosphorylated EGFR. Within 30 seconds of application of the dye-conjugated EGF ligands to cultured cells, three different fluorescent spots of EGF and Shc1 appeared on the cell surface. Binding of a single EGF molecule recruited a single Shc1 to the same spot at an early stage of the incubation. No colocalization of two EGF molecules labeled with different fluorescent colors could be observed, although the colocalization was observed at later stages. However, this colocalization depended on autophosphorylation of EGFR since it was not observed in a kinase-deficient EGFR mutant or in the presence of a tyrosine kinase inhibitor. These results indicate that binding of a single EGF molecule activates a preformed EGFR dimer, consistent with the negative cooperativity of ligand binding to EGFR. The results also indicate that tetramerization and oligomerization of EGFR dimers depends on autophosphorylation of the receptor.

P1042

Bmp6/taz/hippo signalling mediates angiogenesis and Vegf-induced endothelial cell response
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Bone morphogenetic proteins (BMPs) have been suggested to play a role in angiogenesis and vascular disorders. Crosstalk between BMPs and vascular endothelial growth factor receptor 2 (VEGFR2) signaling has been incompletely understood. Our findings demonstrate that BMPs are regulated in normoxic and hypoxic endothelial cells, and in vivo after VEGF-induced angiogenesis or myocardial ischemia. Particularly, BMP2 and BMP6 mediate VEGF-induced endothelial cell response. BMP6 further induces vascular growth and mediates VEGFR2 expression via Hippo/TAZ signaling pathway. BMP6 is the first
member of BMP family found to directly regulate both Hippo signaling pathway, by inducing nuclear localization of TAZ, and angiogenesis. Thus, BMP6 may serve as a potential target for anti-angiogenic therapy.

P1043

**Fgfr2-fgfr3 heterodimer: functional significance in head and neck cancer cells.**

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Head and neck squamous cell carcinoma (HNSCC) is a kind of cancer with high mortality and high metastasis. Many reports pointed out that aberrant FGFR signaling is highly correlated with HNSCC. Fibroblast growth factor receptor (FGFR), a kind of receptor tyrosine kinase (RTK) with 4 members (FGFR1-4) and two isoforms (IIIb&IIIc), plays an important role in cellular migration. However, there is still no effective treatment targeting FGFR. To explore how FGFR regulates HNSCC migration, we conducted wound healing assay and found that overexpression of FGFR3IIIb would decrease migration speed of SAS cells, one of HNSCC cell lines. Besides, we confirmed that SAS is a FGFR2-dominant cell line with lower expression of FGFR3. Plus, some reports indicated that different FGFRs would form heterodimer. We then inferred that FGFR3 may inhibit the downstream signaling through binding with FGFR2 to form FGFR2-3 heterodimer. To verify our hypothesis, we firstly utilized Fluorescence cross-correlation spectroscopy (FCCS) and Co-immunoprecipitation (Co-IP) to prove the existence of FGFR2-3 heterodimer. After confirming their existence, we overexpressed FGFR3 in FGFR2 dominant SAS to form FGFR2-3 heterodimer. With the stimulation of FGF7, FGFR2-specific ligand, we noticed that FGFR2-3 heterodimer will not activate MAPK or AKT pathway signaling using immunofluorescence assay western blot result. Hence, FGFR2-3 heterodimer interferes with FGFR2 signaling. To figure out how FGFR2-3 heterodimer influences FGFR2 signal transduction, we made 3 different truncated-FGFR3 plasmids: point mutation(K508R) FGFR3, intracellular-domain-truncated FGFR3(FGFR3-ΔIntra), and transmembrane-domain-truncated FGFR3(FGFR3-ΔTrans). Our preliminary result showed that STAT pathway could not be activated with FGFR2-3 heterodimer after FGF7 treatment. In addition, membrane/cytosol fractionation was conducted, showing that all three FGFR3 mutants are located on membrane. Thus, FGFR3, even that with only extracellular domains, may stay on the membrane via binding with other FGFRs to form dimers. In the future, we will continue the exploration of FGFR2-3 heterodimer using these FGFR3 mutants. With the hope to unravel its functional significance and to develop therapeutic strategies for HNSCC and other FGFR-related diseases.

P1044

**Cytosolic calcium integrates growth factor signaling during organ development and tunes final organ size**

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Organ development requires the integration of multiple input signals, including chemical, mechanical, hormonal and electrical forces. Signal integration relies on second messenger molecules to multiply these input signals and regulate cell proliferation, apoptosis, and other factors affecting organ growth. In
particular, cytosolic calcium (Ca\textsuperscript{2+}) is a universal second messenger in animals that displays very dynamic behavior. A broad range of stimuli such as insulin and G-protein coupled receptors tune Ca\textsuperscript{2+} dynamics. Yet, how Ca\textsuperscript{2+} signaling controls epithelial morphogenesis remains poorly understood. Here, we used the Drosophila wing imaginal disc as a model system to test the hypothesis that insulin-mediated Ca\textsuperscript{2+} spikes contribute to organ growth regulation, while Gαq-mediated Ca\textsuperscript{2+} waves inhibit growth. Additionally, we propose that tissue-wide increases in baseline Ca\textsuperscript{2+} levels slow growth via a decrease in total cell number to maintain proper organ size and patterning. In support of this proposed regulatory model, we discovered that overexpression of Gαq in the wing disc pouch cells results in tissue-wide Ca\textsuperscript{2+} waves and reduces adult wing size. In contrast, insulin signaling stimulates only localized, cellular-level spikes in cytosolic Ca\textsuperscript{2+} and results in larger wings. To test if Ca\textsuperscript{2+} signaling is necessary for insulin-mediated growth responses, the GCaMP6f sensor was expressed at high levels to sequester free cytosolic Ca\textsuperscript{2+} while expressing a constitutively active version of insulin receptor. We show that sequestering Ca\textsuperscript{2+} while upregulating insulin signaling further enhances wing growth. This suggests the possibility that insulin-mediated Ca\textsuperscript{2+} spikes act to slow growth. Further phenotypic analysis under these conditions shows that there is no change in cell size in the tissue but an increased cell number. In sum, Ca\textsuperscript{2+} signaling provides a negative feedback regulation into hormonal growth regulation and serves as a homeostatic regulator of epithelial growth.

**Kinases and Phosphatases 1**

P1045

**The kinase activity of Drosophila BubR1 is required for insulin signaling-dependent stem cell maintenance**

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As a core component of the mitotic checkpoint complex, BubR1 has a modular organization of molecular function, with KEN box and other motifs at N terminus inhibiting the anaphase-promoting complex/cyclosome, and a kinase domain at the C terminus, whose function remains unsettled, especially at organismal levels. We generate knock-in BubR1 mutations in the Drosophila genome to separately disrupt the KEN box and the kinase domain. All of the mutants are homozygously viable and fertile and show no defects in mitotic progression. The mutants without kinase activity have an increased lifespan and phenotypic changes associated with attenuated insulin signaling, including reduced insulin receptor (InR) on the cell membrane, weakened PI3K and AKT activity, and elevated expression of dFoxO targets. The BubR1 kinase-dead mutants have a reduced cap cell number in female germaria, which can be rescued by expressing a constitutively active InR. We conclude that one major physiological role of BubR1 kinase in Drosophila is to modulate insulin signaling.
Role of putative eIF2-alpha kinases in the Integrated Stress Response of *Entamoeba histolytica*
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*Entamoeba histolytica*, the causative agent of human amoebiasis, faces several challenges during its life cycle imposed by the host’s immune response. These challenges cause cellular stress, with which cells need to cope in order to survive and multiply. Our laboratory has shown that in *E. histolytica*, global protein synthesis is stalled in response to some stresses, and that this is correlated with the phosphorylation of the alpha subunit of a central translation initiation factor, eIF2. There is bioinformatic evidence that *E. histolytica* has at least two eIF2-alpha kinases (EHI_035950; EHI_109700), but further characterization is required. The objective of this project was to test the hypothesis that these two proteins are actual eIF2-alpha kinases and that they are a key factor in the Integrated Stress Response (ISR) of this parasite. First, *E. histolytica* cells were subjected to different stresses, and levels of transcription of the putative eIF2-alpha kinases were measured by RT-PCR. Both kinases exhibited increase transcription in response to heat, serum starvation and ER stresses. However, a similar increase was not evident for oxidative stress. This suggests that these kinases are part of the ISR for a subset of stresses. Next, to authenticate the putative kinases, the active domains were subcloned into yeast expression vectors, and the vectors were transformed into a yeast strain (H1893) that lacks the sole endogenous kinase gene. Authentic eIF2-alpha kinases will phosphorylate endogenous eIF2-alpha, which, in turn, reduces protein translation and cell growth. Yeast strain H1893 showed a significant reduction in growth when EHI_035950 was expressed, suggesting that at least one of the putative kinases is authentic. We used the Trigger System to silence the putative kinase genes. To date, we have achieved partial knockdown (50%) of expression of EHI_109700. Finally, we carried out an *in-silico* analysis of the structural features of the putative kinases. While other parasites have eIF2-alpha kinases with distinctive regulatory domains that allow the kinases to respond only to specific stress signals, such structural features are not evident in *E. histolytica*’s kinases. This might indicate that there is a yet uncharacterized crosstalk between different stress signals and the activation of these enzymes in this pathogen.
phosphoinositide kinase required to generate PIP2 involved in housekeeping functions and Drosophila cells lacking this enzyme are not viable. By contrast, dPIP5K produces a PLC sensitive pool of PIP2 required for sensory transduction in photoreceptors. However, it is presently unclear how these lipid kinases are regulated to generate pools of PIP2 for PLC dependent and independent functions. In this study we show that the \( d\text{PIP5K} \) gene is alternately spliced to generate two protein isoforms (\( d\text{PIP5K}_S \) and \( d\text{PIP5K}_L \)) with a conserved lipid kinase domain but with distinct C-terminal domain. \textit{In vitro}, \( d\text{PIP5K}_L \) showed PI4P 5-kinase activity. While \( d\text{PIP5K}_S \) did not show this activity \textit{in vitro}, it was able to inhibit the PI4P 5-kinase activity of \( d\text{PIP5K}_L \). \textit{In vivo}, we find that \( d\text{PIP5K}_L \) but not \( d\text{PIP5K}_S \) is necessary and sufficient to support PIP2 turnover during phototransduction. By contrast, we find \( d\text{PIP5K}_S \) negatively regulates the function of \( d\text{PIP5K}_L \) \textit{in vivo}. Thus our data demonstrate novel molecular mechanisms by which phosphoinositide kinase activity is regulated during PLC signaling \textit{in vivo}.

\textbf{P1048}

\textbf{Regulation of the Non-Receptor Tyrosine Kinase Ack1 by the Mig6 Homology Region}

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Ack1 (Activated Cdc42-associated kinase) is a non-receptor tyrosine kinase (NRTK) that belongs to the Ack family of kinases. The domain composition of this 120-kDa protein is unique among the NRTKs. Ack1 is the only protein known to possess a region highly homologous to Mig6, a negative regulator of the epidermal growth factor receptor (EGFR). The regulatory mechanisms governing Ack1 activity are likely to be different than those of other known NRTK families. Residues that are important for Mig6 regulation of EGFR are conserved in the Mig6 homology region (MHR) of Ack1. We previously showed that cancer-associated point mutations within the MHR activate Ack1. We hypothesize that (by analogy to Mig6-EGFR) the MHR of Ack1 is engaged in an intramolecular interaction with the catalytic domain, and plays an autoregulatory role. We have identified three Src phosphorylation sites within the MHR of Ack1 (Y827, Y859, and Y860). We used synthetic peptides to probe the specificity of Src and Ack1 toward these tyrosines. Y859 phosphorylation by Ack1 is enhanced by prior phosphorylation of Y860, a site that is preferentially phosphorylated by Src. These results mirror observations for Src-mediated phosphorylation of Mig6, suggesting that the inhibitory mechanisms may be similar. Thus, the existing inhibitory intramolecular interactions between the Ack1 kinase domain and the MHR may be regulated by tyrosine phosphorylation within this region.

\textbf{P1049}

\textbf{Keratinocyte Conditional Reprogramming is independent of Rho Kinase inhibition}

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Epidermal stem cell maintenance and differentiation are tightly coordinated processes. Epidermal stem cell mechanisms have broad applications in wound healing, aging, and cancer research. In cancer, so-called cancer-stem cells may adopt and/or disrupt these stem cell maintenance and differentiation programs to initiate primary tumors, recurrences, and metastases, driving treatment resistance. The Rho Kinases (ROCK1/ROCK2) reportedly regulate differentiation in primary human epidermal keratinocytes. Y-27632, a small molecule ROCK inhibitor, is a component of conditional
reprogramming. Conditional reprogramming requires routine ROCK inhibition to promote long-term keratinocyte proliferation by inducing an epidermal stem cell state. It remains unknown how Y-27632 promotes conditional reprogramming. We hypothesized that ROCK activity prevents keratinocyte proliferation. To test this hypothesis and assess the individual roles of the ROCK1 and ROCK2 in conditional reprogramming, we generated CRISPR-Cas9 ROCK1 and ROCK2 knockouts in a human foreskin keratinocyte (HFK) model. Single ROCK isoform knockout was insufficient to promote HFK proliferation without Y-27632. We next generated ROCK1/2 double knockouts to test whether ROCK double knockouts could mimic conditional reprogramming without Y-27632. ROCK double knockouts also require Y-27632 to proliferate. Furthermore, we observed reduced ΔNp63 protein expression, a marker of keratinocyte stem cells, following Y-27362 withdraw regardless of ROCK1 and/or ROCK2 status. These data suggest that the small molecule inhibitor Y-27632 promotes epidermal stem-ness and proliferation through a ROCK-independent mechanism. Future directions include determining the off-target action by which Y-27632 promotes keratinocyte proliferation and stem-ness. This will yield insights into how epidermal stem cells are maintained, enabling cancer stem cell targeting research.

P1050

**Mad dephosphorylation at the nuclear pore is essential for asymmetric stem cell division**

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Stem cells often divide asymmetrically to generate a stem cell and a differentiating daughter cell. Yet it remains poorly understood how a stem cell and a differentiating daughter cell can receive distinct levels of niche signal and thus acquire different cell fates (self-renewal vs. differentiation) despite being adjacent to each other and thus seemingly exposed to similar levels of niche signaling. In the *Drosophila* ovary, germline stem cells (GSCs) are maintained by short range bone morphogenetic protein (BMP) signaling; the BMP ligands activate a receptor that phosphorylates the downstream molecule Mothers against decapentaplegic (Mad). Phosphorylated Mad (pMad) accumulates in the GSC nucleus and activates the stem cell transcription program. We demonstrate that pMad is highly concentrated in the nucleus of the GSC, while quickly decreases in the nucleus of the differentiating daughter cell, pre-cystoblast (preCB), before the completion of cytokinesis. We also show that a previously identified Mad phosphatase, Dullard (Dd), is required for the asymmetric partitioning of pMad. Our mathematical modeling recapitulates high sensitivity of the ratio of pMad levels to the Mad phosphatase activity and explains how the asymmetry arises in a shared cytoplasm. We reveal a new mechanism for breaking symmetry of daughter cells during asymmetric stem cell division.

P1051

**Shootin1a dephosphorylation by protein phosphatase-1 for netrin-1–induced axon guidance**

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Formation of neural networks require neurons to connect to their synaptic partners with precision. The accurate pathfinding of axons depends crucially on the transduction of extracellular chemical signals to
driving force through the axonal growth cone. At the leading edge of axonal growth cones, shootin1a mechanically couples F-actin retrograde flow and adhesive substrate through an actin-binding protein cortactin and cell adhesion molecule L1-CAM. Furthermore, netrin-1–induced shootin1a phosphorylation by PAK1 enhances the bindings of shootin1a to its interacting proteins, resulting in the promotion of traction force for axon outgrowth. In addition, shootin1a can read the gradients of netrin-1 and regulate the axon turning. However, the molecular mechanism of shootin1a dephosphorylation is uncharacterized. Moreover, how shootin1a dephosphorylation mediates axon guidance remains unknown. To identify protein phosphatases (PPs) for shootin1a dephosphorylation, we used a pharmacological approach to inhibit the phosphatases in cultured neurons and determined the shootin1a phosphorylation level. Treatment of okadaic acid, an inhibitor of PP1/PP2A, increased the phosphorylation level of shootin1a at the leading edge of axonal growth cones. However, shootin1a phosphorylation was unaffected by endothall, a PP2A inhibitor. In vitro dephosphorylation assay showed that PP1 but not PP2A decreased the phosphorylation level of shootin1a at Ser101 and Ser249. In addition, shootin1a was dephosphorylated in PP1-overexpressing COS7 cells. In developing neurons, shootin1a was partially colocalized with PP1 within axonal growth cones. Taken together, these results suggest that shootin1a is dephosphorylated by PP1 in vitro and in vivo. Moreover, we are currently analyzing the effects of shootin1a dephosphorylation by PP1 phosphatase on netrin-1–induced axon guidance. These analyses will help us to understand how growth cones can navigate axonal projection in response to guidance molecules in the brain.

Lysosomes

P1052

**Phagosome resolution regenerates lysosomes and maintains the degradative capacity in phagocytes**
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During phagocytosis, phagocytes like macrophages engulf and sequester unwanted particles like bacteria into phagosomes. Phagosomes then fuse with lysosomes to mature into phagolysosomes, resulting in the degradation of the enclosed particle. Ultimately, phagosomes must be recycled to help recover membrane resources like lysosomes consumed during phagocytosis, a process referred to as phagosome resolution. Little is known about phagosome resolution, which may proceed through exocytosis or membrane fission. Here, we show that bacteria-containing phagolysosomes in macrophages undergo fragmentation through vesicle budding, tubulation, and constriction. Phagosome fragmentation required cargo degradation, the actin and microtubule cytoskeletons, and clathrin. We provide evidence that lysosome reformation occurs during phagosome resolution since the majority of phagosome-derived vesicles displayed lysosomal properties. Importantly, we showed that the clathrin-dependent phagosome resolution is important to maintain the degradative capacity of macrophages challenged with two waves of phagocytosis. Overall, our work suggests that phagosome resolution contributes to lysosome recovery and to maintain the degradative power of macrophages to handle multiple waves of phagocytosis.
P1053

**OCRL: A New Player In Lysosome Positioning And mTOR Activation**

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Lysosomal positioning and mTOR (mammalian target of rapamycin) signaling coordinate cellular response to nutrient levels. Inadequate nutrient sensing can result in degenerative diseases of the brain, eye and kidneys. Mutations in OCRL, an inositol polyphosphate 5-phosphatase, cause Lowe syndrome, a X-linked recessive disease that presents with congenital cataracts, developmental delay and renal dysfunction. Here, by using structured illumination microscopy (SIM) we show that OCRL is localized to the centrosome by its ASH domain. It recruits microtubule anchoring factor SSX2IP to the centrosome and is critical for the formation of the microtubule organization center. Deficiency of OCRL in human and mice cells results in loss of microtubule organization centers and persistent perinuclear lysosome positioning. Single molecule tracking of lysosomes in OCRL deficiency cells revealed impaired microtubule-based lysosome movements, which in turn leads to mTOR inactivation and abnormal nutrient sensing. Centrosome targeted SSX2IP (PACT-SSX2IP) can restore microtubule anchoring and mTOR activity in OCRL deficient cells. Importantly, boosting the activity of mTORC1 by a pharmaceutical activator MHY1485 restores the nutrient sensing ability of Lowe patient-derived cells. Our findings identify a novel function of OCRL and highlight mTORC1 as a novel therapeutic target for Lowe syndrome.

P1054

**Phagolysosomes tubulate into small vesicles to digest cell corpses**

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Phagocytosis is an essential tool for cells to clear waste from their environment, including cell corpses and debris. The elimination of pathogens by phagocytosis is an early response of the innate immune system. Although the mechanisms of phagosome formation and lysosomal fusion are well studied, how phagolysosomal cargos are resolved is poorly understood. Using time-lapse imaging, we tracked the phagolysosome that digests the second polar body, a cell that undergoes programmed necrosis after fertilization. We discovered that the phagolysosome tubulates into small vesicles to facilitate cell corpse clearance. Tubulation occurs after the corpse membrane breaks down inside the phagolysosome and amino acids are released by solute transporter SLC-36.1, which in turn activates TORC1, but not TORC2. Downstream of TORC1, subunits of the lysosome resident BLOC-1-related complex (BORC) activate the Arf-like GTPase ARL-8 for tubulation of the phagolysosome. ARL-8 is thought to link the phagolysosome membrane to motor proteins on microtubules to extend tubules and release vesicles. We found that both dominant negative GDP-bound and constitutively active GTP-locked arl-8 mutants show reduced tubulation of the phagolysosome, revealing that the ARL-8 GTPase needs to cycle to promote tubulation. Altogether, we established an *in vivo* model to follow a single phagolysosome over time, revealed the critical steps of tubulation and vesiculation of large phagolysosomes, and identified the molecular pathway regulating these key steps of phagolysosomal clearance. As phagocytosis is an important part of the innate immune system, vesiculation of large phagolysosomes to degrade cell corpses and pathogens may play a vital role in efficiently protecting cells against engulfed cargos.
A lysosomal hydrolase receptor, CPBF2, is associated with motility and invasion of the enteric protozoan parasite *Entamoeba histolytica*

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Proper targeting and secretion of lysosomal hydrolases are regulated by transporting receptors. *Entamoeba histolytica*, the enteric protozoan parasite responsible for human amebiasis, has a unique family of lysosomal hydrolase receptors, cysteine protease binding protein family, CPBF. CPBFs, consisting of 11 members with conserved domain organization, bind to a wide range of cargos including cysteine proteases (CP) and glycosidases, which are also known to be involved in the pathogenesis of this parasite. Ligands for three members CPBF1, CPBF6, and CPBF8 were previously proven to be CPs, α- and γ- amylases, and β-hexosaminidase and lysozymes, respectively. In this study, we conducted a comprehensive screening of all 11 CPBFs which are involved in tissue invasion. We found that gene silencing of CPBF2 caused significant reduction of Matrigel invasion. Characterization of CPBF2 revealed that it is involved in cell motility and peripheral F-actin formation. Unexpectedly, these roles of CPBF were not related to its cargo, α-amylase. Specific silencing of genes encoding CPBF2 and α-amylase did not affect α-amylase activity and Matrigel invasion, respectively. This is the first demonstration that a putative hydrolase receptor is involved in cell motility and invasion in parasitic protozoa.

Irbit controls autophagy and lysosomal biogenesis by regulating mcoln1 activity

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Inositol 1,4,5-trisphosphate receptor (IP₃R) binding protein released with IP₃ (IRBIT) suppresses the activity of IP₃R in an IP₃ competitive manner, while IRBIT facilitates massive Ca²⁺ transfer from endoplasmic reticulum (ER) to mitochondria by promoting the formation of ER-mitochondria contact sites. Mitochondria are essential cellular organelles for energy production. Impaired mitochondrial Ca²⁺ homeostasis causes a bioenergetic crisis and induces autophagy to restore energy and building blocks necessary for normal growth. However, the role of IRBIT in autophagy has not been characterized. Here, we report that downregulation of IRBIT induces autophagy independently of AMP-activated protein kinase / mechanistic target of rapamycin / Unc-51 like kinase 1 (AMPK/mTORC1/ULK1), a known canonical autophagy pathway. We found that IRBIT depletion induced mitochondrial fragmentation and decreased mitochondrial membrane potential. Autophagosomes were increased in IRBIT depleted cells and were further increased in the presence of lysosomal inhibitor. These results suggested that autophagy is activated to remove damaged mitochondria in IRBIT depleted cells. We identified that IRBIT interacts with lysosomal Ca²⁺ channel mucolipin 1 (MCOLN1). Downregulation of IRBIT activated lysosomal Ca²⁺ release through MCOLN1, which promoted nuclear translocation of transcription factor EB (TFEB), a master regulator of lysosomal biogenesis and autophagy. IRBIT depletion induced upregulation of several autophagy and lysosomal genes, resulting in the increase of proteolytic capacity.
Collectively, IRBIT plays an important role in mitochondrial function and it suppresses over-activation of autophagy and lysosomal function via regulating MCOLN1/TFEB pathway.

P1057

The role of ERAD in cystinosis pathogenesis
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Cystinosis is a genetic disease characterized by the accumulation of cystine within the lysosomal lumen. The causative gene of this disease is CTNS that encodes the protein Cystinosin, a cystine exporter on the lysosomal membrane. Cystinosin exports cystine from the lumen to the cytosol in a symport fashion. Mutations that lead to the absence of a functional cystinosin protein on the lysosomal membrane lead to the toxic buildup of cystine load within the lumen. One possible cause of genetic diseases could be that the mutant protein is less stable than the wild-type counterpart. In this study we aim to explore the role of protein stability in cystinosis pathogenesis. We screened through the different disease-causing mutations and assayed for their half-life in a cycloheximide chase experiment. We identified one mutation, that contains a truncation of 7 amino acids (67ITILELP73), to have a short half-life (<6 hours). We also demonstrated that this mutation is degraded in a proteasome dependent manner and can be stabilized by treating cells with MG132. We also show that the mutation is Endo H sensitive and cannot be enriched in an immunopurification of lysosomes. Furthermore our imaging results show an ER localization of the mutant protein and that a knockdown of the AAA ATPase p97 stabilizes the protein. Together, our data suggests that ERAD plays a role in degradation of this disease causing cystinosin mutation, and further studies to explore the players involved in such a degradation could help us better understand the pathogenesis of this metabolic disease.

Mechanisms of Disease

P1058

Sex differences: transcriptional response to a high fat diet in MuRF1 KO mice
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Background In skeletal muscle, sexual dimorphism in substrate utilization, diet-induced insulin resistance, age-related changes in metabolism, wasting, and muscle response to dietary intake have been observed. In response to high fat feeding, females are generally more protected from deleterious effects than males. As obesity and Type II Diabetes rise globally, it is important to understand the similarities and differences in the response of metabolic tissues between males and females. We wanted to evaluate the impact of sex on the transcriptome of our MuRF1 KO (KO) mice under normal conditions and when challenged by a high fat diet (HFD). Methods RNA was isolated from the gastrocnemius muscle of male and female WT and KO mice that were fed either standard chow (Envigo 2918) or a 45% HFD (Research Diets D12451) for 22 weeks (n = 4). RNA was enriched for mRNA prior to library preparation. RNA sequencing was done using 150 bp paired-end reads (~ 31.6 M reads per sample). Differentially expressed genes (DEGs) were identified using DESeq2 with an FDR set to 5%. Results Compared to their respective WT controls on chow, male KO animals had 1174 DEGs while females had
Twenty-three genes were found to be changed in common. The direction of change was the same between male and female KOs, indicating a common mechanism. Eighty-two DEGs were uniquely changed in females and 1151 were uniquely changed in males. When challenged with a HFD, KO animals showed a greater transcriptional response than their WT counterparts. Males had 1821 DEGs (v. 179 in WT) while females had 4425 DEGs (v. 2090 in WT). In both male and female KO animals, oxidative phosphorylation and ribosomal pathways were most significantly changed, though the direction of change was opposite. **Conclusion** In skeletal muscle, sex highly influences the genes and pathways altered in response to a HFD. Even among common pathways identified, the response between males and females differed. Loss of MuRF1 results in common and unique transcript changes between males and females under normal conditions and, in response to a HFD, results in a greater transcriptional response in skeletal muscle.

**P1059**

Genes deregulated by DUX4-induced oxidative stress: a potential target for future FSHD therapy?  
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Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant disease and the third most common muscular dystrophy in the world. It is associated with the derepression of the *DUX4* gene embedded in the D4Z4 macrosatellite repeat array on chromosome 4. *DUX4* encodes for a transcription factor normally expressed during zygotic genome activation, and its abnormal expression in postnatal muscles is highly toxic. FSHD muscles demonstrate increased levels of oxidative stress markers which correlates with functional muscle impairment, and this is at least partially attributed to *DUX4* expression. *DUX4* is known to cause oxidative stress and DNA damage in muscle cells and can itself be an oxidative stress target. Nevertheless, the exact role of oxidative stress in FSHD is yet unclear and direct antioxidant treatment does not prove effective for FSHD patients, highlighting the need for alternative approaches to address the consequences of oxidative stress in FSHD muscles. One of these approaches would be to target the genes deregulated by DUX4 through oxidative stress, rather than targeting the oxidative stress itself. We used immortalized human myoblasts expressing *DUX4* (MB135-DUX4) To identify oxidative stress sensitive DUX4 targets. These cells exhibit higher levels of reactive oxygen species (ROS) than normal MB135 myoblasts and do not differentiate properly unless treated with antioxidants. The transcriptome analysis of antioxidant-treated MB135 and MB135-DUX4 myoblasts revealed 182 genes deregulated by DUX4 but normalized upon antioxidant treatment. Muscle tissue development was the most significant overrepresented term with 10 genes falling into this category including *PITX1*, the gene previously linked to FSHD. Another enriched category was cellular response to inorganic substances (7 genes) represented by the genes encoding for metallothioneins (MTs). Here we explored the contribution of *PITX1* and MTs to the oxidative stress-induced muscle differentiation defects in FSHD. We revealed that *PITX1* is regulated by oxidative stress and have shown that the silencing of this gene partially restored the differentiation capacity of MB135-DUX4 myoblasts.
Proteolytic mechanisms involved in muscle wasting in spawning threespine stickleback  

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In fish, skeletal muscle serves as a protein store and protein oxidation accounts for the most important source of energy. Proteins could be readily degraded onto amino acids by endogenous proteolytic systems, such as lysosome, calcium-dependent (or calpain), and ATP-dependent ubiquitin-proteasome, operating in vivo both in fish and mammals. However, their relative importance in total protein degradation as well as protease activity patterns during skeletal muscle wasting in fish and mammals are clearly distinct and not fully understood yet. Threespine sticklebacks, G. aculeatus, starve and expend much energy during spawning migration and gonad maturation and lost up to one-fourth of their body weight mostly due to protein loss by the end of the spawning season. The effect of muscle exhaustion revealed by myotube diameter and immunofluorescent staining of structural proteins was found to be more pronounced in female stickleback lost up to 55% of their muscle protein reserves. To probe mechanisms involved in muscle wasting, the major proteolytic systems were enzymatically assayed. It turned out that across the spawning period, the lysosomal autophagy activated up to 4-fold primary due to cathepsin D as well as calpain activity substantially (up to 7,5-fold) increased in the skeletal muscle of fish. In contrast to mammals, the proteasome activity could not be considered as the major path of protein degradation involved in skeletal muscle loss in fish as it did not significantly change in migrating and spawning stickleback. Summarizing, autophagy and calpain-dependent pathways activation is a mechanism of mobilization of muscle protein depot to maintain individual viability of migrating and spawning stickleback. The study was supported by the budget funding by theme 0218-2019-0076, and RSF grant 19-14-00092.

Role for mitochondrial-ER interactions in megaconial congenital muscular dystrophy, a disorder produced by phospholipid imbalance

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Mutations of choline kinase beta (CHKB), the enzyme that catalyzes the initial step in de novo phosphatidylcholine (PC) biosynthesis, cause megaconial congenital muscular dystrophy (MDCMC). Although MDCMC is a congenital muscular dystrophy, the impact of this disorder extends beyond the motor domain. The disease is characterized by muscle wasting, weakness from early infancy, severe intellectual disability and cardiac dysfunction. The histological hallmarks of MDCMC are abnormally enlarged mitochondria that accumulate at the cell periphery and are depleted in central regions referred to as “cores”, primarily in type II fast-twitch muscle fibers. Moreover, defects in mitochondrial
respiratory function are evident at later stages of disease progression. Since muscle degeneration occurs predominantly in muscle fibers with central cores, muscle dystrophy is linked to the mitochondrial distribution defects. It is currently unknown, though, how a defect in PC biosynthesis can lead to altered mitochondrial distribution and muscular dysfunction. We find that lipid imbalance produced by defects in CHKB also affects ER: ER exhibit defects in morphology and distribution and co-localize with megaconial mitochondria in skeletal muscle from the rmd mouse model for the disease and from a MDCMC patient. Lipid imbalance also results in activation of the unfolded protein response (UPR), a conserved signaling pathway activated by ER stress (accumulation of unfolded proteins in ER). Specifically, we detect up-regulation of the binding immunoglobulin protein (BiP) in rmd mice at 3 weeks of age, prior to the onset of muscular dystrophy. We developed a cell culture model for the disease using the C2C12 cell line. Inhibition of choline kinase in C2C12 myotubes results in up-regulation of UPR markers and Ca^{2+} leakage at ryanodine receptors (abundant integral ER ion channels that mediate excitation-contraction coupling in skeletal muscle), as well as elevated Ca^{2+} levels in and ROS production by mitochondria. Our data points to ER stress and defects in Ca^{2+} homeostasis in ER and mitochondria as early events during disease progression and may therefore be an underlying cause of the disease.

P1062

Ibmpfd related vcp mutations and pathogenesis are associated with defects in a tubular lysosomal network
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Valosin containing protein (VCP) is a highly conserved, hexameric AAA ATPase, associated with a plethora of cellular activities including gene expression, protein homeostasis, stress response, and organelle biogenesis among many others. The protein consists of two ATPase domains - D1 and D2 and a regulatory co-factor binding N terminal domain. Mutations in VCP are associated with a variety of degenerative diseases inclusive of Inclusion body myopathy with Paget’s disease of the bone and frontotemporal dementia (IBMPFD). Patients exhibit muscle, bone and neuronal degeneration with a varying degree of clinical pathology and penetrance. Multiple VCP mutations are associated with IBMPFD and understanding how each mutation affects the cellular functions of VCP could allow us to better predict clinical outcomes and design personalized treatment options. Over-expressing VCP patient mutations in Drosophila has been shown to mimic many pathologies observed in human patients, suggesting that fly models could be useful for studying VCP disease pathogenesis. Using CRISPR, we have generated individual Drosophila knock-in mutants for the Ter94 protein, the VCP homologue in flies, that include 9 of the most prevalent and/or severe IBMPFD mutations. Our Drosophila models display progressive degenerative phenotypes that are on par with many clinical features of IBMPFD. Using these models, we are studying the effect of each patient mutation on lysosomes and its impact on the autophagy-lysosome pathway owing to the fact that increased protein aggregation is a major cellular hallmark of VCP diseases. Previously, we found that VCP is required for the maintenance and integrity of a tubular lysosomal (TL) network that spans across the body wall muscles in Drosophila and knock down of VCP results in complete disruption of this lattice structure. Significantly, only a subset of VCP patient mutations negatively affect TLs, underscoring the importance of studying each mutation individually. In the long term, we hope these studies will shed light on the pathological outcomes of individual VCP patient mutations and inspire new treatment strategies. This project is supported by NIH grant R00NS100988.
Mechanotransduction in Tissue Homeostasis and Disease

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Mechanical competition triggered by innate immune signaling drives the collective extrusion of bacterially-infected epithelial cells

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Several intracellular bacterial pathogens alter the mechanics of their mammalian host cells to promote their dissemination through tissues. Conversely, host cells may respond to the presence of intracellular pathogens by altering their own mechanical behavior to limit infection spread. Here, we monitored epithelial cell monolayers sparsely infected with the intracellular bacterial pathogens *Listeria monocytogenes* or *Rickettsia parkeri* over the course of several days. We found that, under conditions where these pathogens trigger innate immune signaling through the NF-kB pathway and use actin-based motility to spread non-lytically from cell to cell, domains of infected cells form enormous three-dimensional mounds. We show that these mounds result from uninfected cells moving toward the site of infection, collectively squeezing the softer and weaker infected cells upward and ejecting them from the epithelial monolayer. Bacteria in mounds are less able to spread laterally in the monolayer, limiting the growth of the focus, while extruded infected cells eventually undergo cell death. This cellular competition between infected and uninfected cells leading to infected cell extrusion en masse is driven by active directional migration in uninfected bystander cells, intact cell-cell junctions and innate immune signaling. In addition, cells in infected monolayers exhibit behavioral and molecular signatures of the epithelial to mesenchymal transition (EMT). In summary our findings showcase that coordinated forceful action by uninfected bystander cells actively eliminates large domains of infected cells, consistent with the hypothesis that this collective cell response represents an innate immunity-driven process that could help to limit the local spread of infection.

P1064

Jagged1 is mechanosensitive and regulates, with Vimentin, the arterial remodeling response to hemodynamic stress

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The main causes of death in the world are cardiovascular diseases such as cardiac ischemia and stroke. To find new pharmacological targets, and to develop new treatments, it is important to get a better understanding of the molecular mechanisms underlying vascular diseases. These pathologies have as a common feature, the disruption of the normal blood flow and vessel homeostasis. Under these circumstances, the elasticity and functionality of the arteries are compromised. Mice in which the intermediate filament Vimentin has been knocked-out display disrupted vascular smooth muscle cell (VSMC) differentiation and abnormal vascular remodeling. Vascular remodeling is regulated by signaling between endothelial and vascular smooth muscle cells through the Notch signaling pathway. Upon
shear stress, Vimentin is phosphorylated at serine 38 and interacts with the Notch receptor ligand Jagged1. This interaction enhances ligand-receptor trans-endocytosis and increases Jagged1 signaling strength. Shear stress increases Jagged1 and soluble Vimentin protein levels, induces Jagged1 re-localization from the membrane into cytosolic clusters, and enhances the signal sending potential of endothelial cells. Protein and gene levels of Jagged1 are sensitive to changes in the amount of shear stress. Jagged1 inhibition reduces ERK1/2 mechanical activation. Taken together, these findings demonstrate that Vimentin and Jagged1 forms a central part of a mechanochemical transduction pathway that regulates multilayer communication and structural homeostasis of the arterial wall.

P1065

Cellular mechanosensation determines immunological sensitivity of STAT6-mediated alternative macrophage activation
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Macrophages play an important role in inflammation and immune responses, and their malfunctioning is associated with the progression of chronic human diseases such as cardiovascular disease, obesity, and cancer. There are multiple functional states of macrophage activation induced by various immune pathways, including the most well-known contrasting phenotype, pro-inflammatory (M1) and alternative (M2) activation. Although macrophages are located in various tissues exposed to different physical environments, however, how the extracellular mechanical signals determine their immunological activation remains unclear. Here, we demonstrate that immunological activation of bone marrow-derived macrophage (BMDM) is precisely controlled in response to cellular mechanosensation of substrate compliance. As the substrate stiffness decreases, BMDMs are poorly spread with decreasing F-actin content and show reduced expression of arg-1 which are triggered by induced M2 activation. Moreover, nuclear translocation of STAT6, key transcription factors of activation pathways, is controlled by substrate stiffness, and vertical compressive forces, which potentially increases nuclear stresses, also promotes nuclear localization of transcription factors. We also find that protein expression of arg-1 induced by macrophage activation is tightly coupled with the nuclear translocation of STAT6. These results reveal that actomyosin contractility-mediated cell spreading can act as an immuno-regulatory pathway to determine the immunological sensitivity of macrophage to the physical properties of the extracellular microenvironment, providing a new perspective on the relationship between physical cues and immune responses.

P1066

Epithelial cells recover their substrate adhesion through retraction fiber-guided lamellipodia
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The integrity of epithelia is constantly challenged during developmental, mitotic, and pathological processes. Here, we used acute chemical and mechanical perturbations to examine how epithelial cells respond to partial delamination events that threaten tissue integrity. We saw that detached cells remained tethered to the substrate via long membrane tubules, termed retraction fibers, that could extend multiple cell lengths in seconds. Cells then extended lamellipodia between adjacent retraction
fibers to rapidly and efficiently reestablish substrate adhesion. Although retraction fibers had been observed to guide readhesion following cell division, their general role facilitating seconds-scale cellular reattachment had, to our knowledge, been overlooked. We suggest that retraction fiber-templated respreading may represent a general mechanism to reestablish tissue integrity in the face of acute disruption, both during normal tissue homeostasis as well as in a number of pathological scenarios.

P1067

Dystrophin Deficiency Leads to Altered Tension Transmission Via Focal Adhesions in Muscle Cells

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The dystrophin-glycoprotein complex (DGC) is a muscle adhesion complex that anchors the cytoskeleton to the extracellular matrix (ECM), where dystrophin is the cytolinker component of the DGC to the ECM. The absence of dystrophin results in Duchenne muscular dystrophy (DMD), a lethal muscle wasting disease in which the muscle cell membrane becomes fragile and ruptures due to the loss of the cytolinker. Interplay between disruption of the DGC and regulation of focal adhesions components and signaling has been previously shown, such as upregulation of α7β1 integrins and paxillin, as well as aberrant signaling of the survival MAPK signaling pathway. We hypothesized that loss of dystrophin would decrease adhesion strength of muscle cells to the ECM and would thus lower forces sensed at focal adhesions. Here we show that dystrophin deficient muscle cells transmit less tension via focal adhesions. Using a vinculin bioluminescent tension sensor, we measured focal adhesion tension in transgenic muscle cells expressing wild type (WT) dystrophin and two dystrophin missense mutants - L172H and L54R - which cause decreased levels of dystrophin. We found that cells harboring WT dystrophin showed significantly higher tension across vinculin, compared to dystrophic cells expressing mutant dystrophins. Interestingly, focal adhesion morphology was similar across cells regardless of the type of dystrophin expressed. Moreover, dystrophic cells had decreased activation of ERK1/2, concomitant with the cellular levels of dystrophin. Our results show that expression of mutant dystrophins lead to decreased tension sensing and aberrant mechanotransduction via focal adhesions, compared to cells expressing WT dystrophin. Our data suggests that dystrophin levels may be the main factor leading to tension sensing dysregulation, likely due to weaker cytoskeletal-ECM coupling of the adhesion protein complexes. By determining how dystrophin deficiency dysregulates mechanotransduction, we can aim to better understand DMD disease progression at a molecular level to design more efficient therapies.

P1068

Augmenting Ultrasound-Mediated Cancer Cell Apoptosis using Microtubule Destabilizing Agents

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* Denotes equal work contribution Recently, ultrasound-mediated cancer cell killing has gained widespread attention. However, concerns were raised about normal tissue damage due to local heating.
causing limitations. Studies have indicated susceptibility of cancer cells to mechanical forces in comparison to their normal cell counterparts, resulting in apoptosis upon mechanical activation termed as mechanoptosis. Herein, we report that low frequency ultrasound-induced cancer cell apoptosis is augmented using the microtubule destabilizing agents at cytostatic drug concentrations. Surprisingly, we observed that ultrasound irradiation alone causes microtubule disruption specifically to cancer cells, sparing the normal cells. We provide evidence that microtubule disruption occurs due to calpain protease activation by ultrasound mediated-calcium influx through mechanosensitive Piezo1 channels. Microtubule disruption enhances myosin IIA-regulated contractility through activation of GEF-H1 and RhoA/ROCK pathway which is indispensable to facilitate ultrasound-induced apoptosis. Furthermore, ultrasound promotes Piezo1 expression as well as myosin IIA mediated contractility dependent-Piezo1 localization to the peripheral adhesions, allowing calcium influx by activated Piezo1 to continue the feedback loop. Thus, ultrasound in combination with class of microtubule disruptors can potentially aid in cancer treatment by minimizing drug related side-effects.

P1069

Lamellipodin links Rac to intracellular stiffening and mechanosensitive cell cycling

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Cells exhibit pathological behaviors in response to increased extracellular matrix (ECM) stiffness, including increased cell proliferation and intracellular stiffness. Paramount to the transduction of ECM stiffness into relevant biochemical signals and resultant cellular behaviors is the dynamic remodeling of the actin cytoskeleton. Lamellipodin, a phospholipid- and actin-binding protein and dominate regulator of peripheral actin dynamics in cellular migration has previously been suggested to regulate stiffness-dependent cyclin D1 (a putative early G1 cyclin) induction. However, the extent to which lamellipodin regulates stiffness-sensitive cell cycling and associated intracellular stiffening, whether lamellipodin itself is regulated by ECM stiffness, and how lamellipodin is regulated remain poorly understood. Using fibronectin-coated, low (soft) and high (stiff) stiffness polyacrylamide hydrogels that mimic healthy and pathological femoral artery wall stiffnesses in vitro, respectively, and seeded with 10% serum stimulated post serum-starved mouse embryonic fibroblasts (MEFs), we examined differential expression changes in lamellipodin and cyclins using RT-qPCR and immunoblotting. Additionally, we examined the effects of lamellipodin knockdown on intracellular stiffness using atomic force microscopy. Here we show for the first time that lamellipodin itself is significantly regulated by ECM stiffness, in which both actin-mediated intracellular stiffening and Rac1 are coessential to its induction. Furthermore, we show that sustained mechanosensitive lamellipodin is vital across the cell cycle, not only directly regulating the induction of cyclin D1 in early G1, cyclin A2 in S and G2 phases, and cyclin B1 in late G2 and mitosis, but also regulates cell cycle-associated intracellular stiffening. Intriguingly, we bring to light a peculiar observation in which lamellipodin, thought to play a key role in cell protrusion and migration, dominantly regulates cyclins in the nucleus. More imperatively, not only is lamellipodin a mechanistic linchpin between ECM stiffness and downstream stiffness-dependent functions, but, too, is itself mechanosensitive through a newly defined Rac-lamellipodin signaling module essential to the regulation of mechanosensitive cell cycling and intracellular stiffening.
Tissue stiffness regulates pro-inflammatory signaling through interleukin-1 receptor endocytosis
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Interleukin-1 (IL-1) signaling, a potent pro-inflammatory signaling pathway that modulates innate and adaptive immunity, can result in a plethora of autoimmune diseases and cancer if left unchecked. Efforts to understand the dynamics of IL-1 signaling have largely focused on canonical signaling molecules that mediate the cell’s response to IL-1 stimulation. However, recent studies highlight the key role of cell-extracellular matrix interaction in regulating such inflammatory responses, yet the mechanism is unknown. To understand how cell-extracellular matrix interaction regulates inflammatory signaling, we first tested the contribution of integrin-based focal adhesions and the cell’s cytoskeleton. We found that focal adhesions do not directly regulate IL-1 signaling. However, moderate suppression of myosin contractility downregulated IL-1 signaling and decreased the amount of IL-1 receptor on the plasma membrane, suggesting that tension within the cell cortex regulates IL-1 signaling through IL-1 receptor. We showed that the expression of IL-1 receptor does not depend on cortical tension. Conversely, inhibition of dynamin completely rescued the amount of IL-1 receptor and downstream signaling events in cells with suppressed cortical tension, suggesting that cortical tension modulates IL-1 signaling through endocytosis of its surface receptor. Furthermore, we locally modulated cortical tension by plating cells on patterned substrates of defined shapes and showed that IL-1 receptor is consistently enriched in areas of high cortical tension. Inhibition of endocytosis abolished heterogenous receptor distribution on patterned substrates, indicating that cortical tension provides local regulation of IL-1 receptor endocytosis. To understand the biological role of cortical tension-regulated IL-1 signaling, we cultured cells in 3D collagen scaffolds of physiological and pathological stiffnesses. We found that stiff (pathological) microenvironments, which increase cortical tension, upregulated the amount of surface IL-1 receptor in fibroblasts. Moreover, local tissue stiffening induced by mechanical coupling between cells was sufficient to increase the amount of surface IL-1 receptor. Together, our results demonstrate that tissue stiffness facilitates IL-1 signaling through cortical tension-mediated endocytosis of cell surface IL-1 receptor. This elucidates a mechanistic link between mechanical properties of tissue and pro-inflammatory signaling.

Microtubule Nucleation

A hydrodynamic instability drives protein droplet formation on microtubules to nucleate branches

Branching microtubule nucleation is critical for spindle assembly, where it is required to generate microtubules in the spindle for kinetochore fiber tension, spindle bipolarity, and cytokinesis. The nucleation of a new microtubule from the side of a preexisting microtubule requires the protein TPX2, the augmin complex, the γ-tubulin ring complex, and tubulin. The first component to bind to the preexisting microtubule is TPX2, which forms a liquid-like condensate on the microtubule that then recruits the other necessary factors. Other microtubule-associated proteins also form condensed phases.
on microtubules, such as Tau, BugZ, and LEM2. Yet, how condensed proteins behave on the microtubule and how this behavior translates to biological function remain unexplored. We investigated how the dynamics of condensed TPX2 on the microtubule is important for nucleating branches. Using fluorescence, electron, and atomic force microscopies and hydrodynamic theory, we show that TPX2 on the microtubule reorganizes according to a classic fluid mechanics phenomenon known as the “Rayleigh-Plateau instability,” like dew drops patterning a spider web. After uniformly coating microtubules, TPX2 forms regularly spaced droplets in which γ-tubulin ring complexes cluster, and from which branches nucleate. Finally, a stochastic model shows that droplets make branching nucleation more efficient by confining the space along the microtubule where the multiple necessary factors colocalize to nucleate a branch. Taken together, our experiments, theory, and modeling reveal the mechanism by which condensed TPX2 forms droplets on microtubules and the benefit of droplet patterning for branching nucleation efficiency. Our work constitutes the first characterization of the Rayleigh-Plateau instability at the nanoscale of molecular cell biology. We hypothesize that such fluid mechanics plays a role in diverse parts of the cell where condensed phases interact with other cellular filaments in addition to microtubules, such as actin, RNA, and DNA.

P1072

Identifying the In Vivo Role of Non-centrosomal Microtubule Organizing Centers During Cell Migration
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The microtubule (MT) cytoskeleton plays key roles in cell migration via intracellular signaling and modulating cell adhesions and polarity. Within a cell, MTs emanate and grow from microtubule organizing centers (MTOCs), and the localization of an MTOC helps drive specific MT functions. In cultured migratory cells, the MTOC is canonically located at the centrosome with MTs growing towards the leading edge of the cell. In vivo cell migration studies, however, have identified variations in the MTOC site and MT organization, such as MTs radiating towards the trailing edge of the cell from the centrosome, or a broad MTOC at the trailing edge with MTs radiating towards the leading edge. The molecular factors governing the MTOC site, and the impact of MT organization on the migratory functions, in vivo is unclear. During C. elegans development, the Sex Myoblasts (SM) are a pair of cells that undergo a long distance migration to the gonad. Utilizing the SM cells as an in vivo model, we are investigating the presence and function of non-centrosomal MTOCs in cell migration via live confocal imaging of intact animals. Through SM-specific, temporal and spatial MT degradation, we have found an essential role for MTs in SM cell migration. Upon MT degradation, the SM cells fail to fully migrate to the gonad. In addition, directional analysis of growing MTs has identified arrays of MTs running parallel to migration and a MTOC localized near the leading edge of the cell. In contrast to the canonical MT organization, this leading edge MTOC does not appear to be associated with the centrosome. These results highlight a MTOC and pattern of MT organization not typically associated with migratory cells. Further investigation into the significance of the MTs at the leading edge in association with cell adhesions and polarity proteins will provide insights into the mechanisms governing migration in vivo.
MACET4 is a novel microtubule nucleator that regulates microtubule dynamics in the phragmoplast
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Cytokinesis is the final stage of cell division in which cytoplasm is partitioned between daughter cells. The constriction mechanism of cytokinesis adapted by animal cells is not feasible in plants owing to the rigid cell wall. Plants evolved a unique mechanism, which relies on building a partition between daughter cells known as the cell plate. Cell plate assembly starts from the cell center and the expands outward to join the parental cell wall. Although cytokinetic machinery in both plants and animals depends on microtubules, the dynamicity of microtubules in each system differs: microtubules are highly stable in the animal cytokinetic structure, the mid-body, and highly dynamic in the plant structure - the phragmoplast. Despite these differences, the midbody and the phragmoplast share many microtubule-binding proteins. Therefore, it remains obscure which factors define phragmoplast dynamicity. Our goal is to identify and characterize plant-specific proteins in the phragmoplast. To this end, we have identified an embryophyte-specific protein, MACERATOR4 (MACET4) that accumulates at the microtubule nucleation regions of the phragmoplast as well as the cortical division zone. We show that MACET4 binds microtubules in vivo and in vitro, promotes microtubule polymerization at subcritical tubulin concentrations, and decreases lag phase in microtubule bulk polymerization assays. In vitro MACET4 inhibits microtubule rescue resulting in shorter microtubules. Furthermore, we show that MACET4 forms oligomers and induces aster formation in vitro in a manner that is similar to aster formation mediated by centrosomes and TPX2. Transient expression of MACET4 driven by its native promoter in tobacco leaf pavement cells results in labeling of shrinking plus- and minus-ends, increases the frequency of catastrophes, and most notably, colocalizes with microtubule nucleation sites. Accordingly, MACET4 knockdown results in longer phragmoplasts in Arabidopsis root cells. We conclude that MACET4 has direct activity on microtubule nucleation and can regulate phragmoplast length.

PTRN-1/ Patronin-proximal proteins VAB-10B and WDR-62 are required for non-centrosomal microtubule organization in differentiated epithelial cells
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Microtubules are grown and localized from their minus ends at microtubule organizing centers (MTOCs). In differentiated cells, MTOC function is often reassigned from centrosomes to to non-centrosomal sites (ncMTOCs). Despite the ubiquity of ncMTOCs across cell types and organisms, the components required for microtubule growth and localization remain largely unidentified. To address this gap in knowledge, we developed biotin-based proximity labeling in living C. elegans with TurboID/miniTurbo, fast-acting biotin ligase mutants that label proximal proteins for subsequent identification. By expressing a transgene of TurboID fused to the microtubule minus end protein PTRN-1 in differentiated intestinal cells, we spatially enriched in vivo biotinylation labeling to ncMTOCs at the apical membranes. We identified 69 proteins proximal to PTRN-1 and focused on two conserved proteins: VAB-10, a spectraplakin whose orthologs have non-centrosomal microtubule functions, and WDR-62, a protein we
identify as a homolog of vertebrate WDR62, mutations of which are associated with primary microcephaly. Using endogenous localization and depletion studies, we found that VAB-10B and WDR-62 independently regulate the growth and localization of non-centrosomal microtubule arrays; depletion of VAB-10B results in disorganized microtubules and delayed localization of a microtubule nucleation complex γ-TuRC while loss of WDR-62 decreases microtubule numbers and abolishes γ-TuRC localization. These changes in ncMTOC formation occur downstream of cell polarity as the apical polarity protein PAR-3 is properly localized following depletion of VAB-10B or WDR-62. Together our data suggest a division of labor model where microtubule anchorage and nucleation are regulated by distinct complexes. Moreover, as this is the first report for non-centrosomal roles of WDR62 proteins, our study expands the basic cell biological role of this important disease protein.

P1075

Msps governs acentrosomal microtubule assembly and reactivation of quiescent neural stem cells
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The ability of stem cells to switch between quiescence and proliferation is crucial for tissue homeostasis and regeneration. Drosophila quiescent neural stem cells (NSCs) extend a primary cellular protrusion from the cell body prior to their reactivation. However, the structure and function of this protrusion are not well established. In this study, we show that in the primary protrusion of quiescent NSCs microtubules are predominantly acentrosomal and oriented plus-end-out, distal to the cell body. We have identified Mini Spindles (Msps)/XMAP215 as a key regulator of NSC reactivation and acentrosomal microtubule assembly in quiescent NSCs. We show that E-cadherin, a cell adhesion molecule, is localized to NSC-neuropil contact points, in a Msps-dependent manner, and is intrinsically required for NSC reactivation. Our study demonstrates a novel mechanism by which Msps-dependent microtubule assembly in the primary protrusion of quiescent NSCs targets E-cadherin to NSC-neuropil contact sites to promote NSC reactivation. We propose that the neuropil functions as a new niche for promoting NSC reactivation, which may be a general paradigm in mammalian systems.

P1076

Alpha-fodrin is vital to the recruitment of gamma-tubulin to the centrosome
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Fodrin, a cortical cytoskeletal protein associates with the γ-TuRCs, the major microtubule nucleator in cells. Fodrin is a heterodimer of alpha and beta subunits. Through mutliple in vitro approaches we understood that α-fodrin associates through a GRIP-like motif in its C-terminus to γ-tubulin. Further, it regulates the nucleation activity of the γ-TuRCs. We have also found that α-fodrin’s depletion from glioblastoma cells results in a delayed recruitment of γ-tubulin to the centrosome. This manifested into compromised centrosomal activity. We observed reduced presence of γ-tubulin at the centrosome. The centrosomal availability of CDK5RAP2, another centrosomal protein was also significantly reduced. This resulted in reduced microtubule formation in both interphase and mitosis. We also performed a comprehensive analysis to understand the effect of fodrin in cells, through global proteome analysis of fodrin depleted cells and compared them to control cells. It was found that α-fodrin depletion resulted
in an upregulation of cytoskeletal regulatory pathways and apoptosis. Interestingly some microtubule motor proteins such as KIF2B, KIF3C and KIF23 were reduced as a result of α-fodrin depletion. KIF2B has been reported in the literature to be crucial for chromosome movement on the microtubules. KIF23 is important in the antiparallel sliding movement of microtubules to maintain optimal spindle length. In the present study, we found that α-fodrin depleted U-251 MG cells showed reduced interpolar distance of the spindle which is probably affected through the reduced presence of KIF23. KIF3C is a member of KIF3 family proteins that has been reported to facilitate the movement of vesicles through axons for neurite building. α-fodrin is vital to neurite building as reported by other groups. Through this present study, we can conclude that fodrin being a cortical protein is important in various cellular functions besides supporting the plasma membrane. It is an important regulator of microtubule nucleation. It is significant in maintaining the optimal concentration of γ-tubulin and CDK5RAP2 at the centrosome and thereby preserving the centrosome functionality to form microtubules in interphase and mitosis. It is also vital to the activity of key kinesins and there recruitment functions.

P1077

Designing artificial microtubule nucleus by DNA nanotube

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Microtubules (MTs) are hollow cylindrical biopolymers commonly composed of 13 parallel tubulin protofilaments (PFs) and play essential roles in many cellular processes, such as molecular transport, chromosome segregation, cellular organization, and cell motility. The structures of microtubules are critical in those cellular functions. In living cells, the PF numbers and arrangements are tightly regulated by a template protein complex, gamma-tubulin ring complex (γ-TuRC). In contrast, the structure of in vitro MTs has been found to have a broad distribution of PF numbers and helicity. Consequently, single-molecule studies over many decades have ignored the role of MT structures to their cellular functions. To bridge this gap between in vitro and in vivo experiments, well-defined assays are necessary, wherein the MT structures can be engineered. We have developed a DNA origami seed to engineer precise number and spatial configuration of α,β−tubulins. Here, we demonstrate that folding a 24-helix DNA origami rectangle resulted in hollow DNA origami nanotubes. we pattern 12 α,β−tubulins on one of the edges of the DNA origami nanotubes. Preliminary time-lapse microscopy images show that the origami seeds are positioned at the end of MT. Therefore, the DNA origami seeds appear well-suited to nucleate and control the growing MT structures. An in vitro system for controlling MT structures will open a new frontier in studying biochemical and mechanical properties of MT’s in their native structures and designing bio-inspired adaptive structures from hybrid abiotic and biotic polymers. In the meeting, we will also discuss the method to control the orientation of tubulin on DNA origami nanotube.

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Akap6 orchestrates the nuclear envelope microtubule organizing center (mtoc) by linking golgi and nucleus via Akap9

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Non-centrosomal microtubule organization (ncMTOC) is a hallmark of many differentiated cell types. During differentiation of striated muscle cells, both skeletal and cardiac, as well as of osteoclasts, centrosomal proteins and Golgi are localized to the nuclear envelope. Concomitant with this, the main MTOC activity of these cells is found at the nuclear envelope. Still, the molecular composition and regulation of nuclear envelope MTOC is poorly understood. Here, we identify AKAP6 as key component of the nuclear envelope MTOC. By binding both nesprin-1α and Pcnt and AKAP9 through its spectrin domains, AKAP6 anchors the Golgi and centrosomal proteins at the nuclear envelope to assemble the ncMTOC. Several lines of evidence support this conclusion. First, AKAP6 is required and sufficient for centrosomal protein recruitment to the nuclear envelope. Second, AKAP6 and AKAP9 form a protein platform tethering the Golgi to the nuclear envelope. Around the nucleus of rat neonatal cardiomyocytes, both Golgi and nuclear envelope, exhibit MTOC activity utilizing different microtubule-regulating factors. While at the Golgi, microtubule nucleation is dependent on AKAP9, possibly with the help of ninein, at the nuclear envelope both γ-TuRC binding proteins AKAP9 and Pcnt are required. Importantly, AKAP6 is not only required for cardiomyocyte ncMTOC formation, but it is also required for formation and activity of the nuclear envelope MTOC in human osteoclasts, indicating that AKAP6 has a general role in mammalian ncMTOC establishment at the nuclear envelope. Furthermore, ectopic expression of nesprin-1α and AKAP6 is sufficient to recruit endogenous centrosomal proteins to the nuclear envelope of epithelial human cells. Finally, we show that the AKAP6 and AKAP9-dependent Golgi localization at the nuclear envelope is required for cardiomyocyte hypertrophy and that AKAP6 is required for proper osteoclast bone resorption activity. Collectively, we decipher the MTOC at the nuclear envelope as a bi-layered structure generating two different pools of microtubules with AKAP6 as a key organizer.

Mitochondrial Architecture

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Mitochondrial RNA granules are fluid subcompartments of mitochondria, distributed by network dynamics.

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Mitochondrial gene expression is essential for mammalian ATP-production through cellular respiration, and its malfunction underlies many severe human diseases. Upon transcription, mitochondrial RNA was found to form foci that also accumulate multiple RNA-interacting proteins. Various roles have been proposed for these so called mitochondrial RNA granules (MRGs) but their true relevance and precise function remain elusive to date. To solve this problem it is essential to elucidate the mechanisms of assembly and organisation of MRGs. We hypothesised a fluid organisation of MRG-components and found evidence in accordance with a model of liquid-liquid phase separation to underlie MRG formation inside mitochondria, similar to other cellular RNA-protein granules. Furthermore, we investigated the dimensions and internal architecture of these nanoscopic cellular sub-sub-compartments via superresolution microscopy, and show that mitochondrial network arrangement is important for granule distribution. Together, our data provide novel insights on the organisation of MRGs and their interplay with the mitochondrial ultrastructure.
Mechanical interaction of F1FO ATP synthase with lipid membranes

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The shape of biological membranes is constantly remodeled and maintained out of equilibrium by active proteins. The functional capacity of membrane deformation is mainly determined by the mechanical interplay between protein activity and bending elasticity. In our experiments, we find that ATP synthase, a rotating membrane protein that synthesizes the biochemical energy in cells through proton-pumping activity across the membrane, promotes localized nonequilibrium membrane fluctuations when reconstituted in giant lipid vesicles. This study was mainly based on the formation of giant unilamellar vesicles (GUVs) through electroformation technique followed by mild detergent protein reconstitution. Fluctuations of proteoGUVs were filmed and analyzed by flickering spectroscopy. We find that ATP synthase rotates at a frequency of about 20 Hz, promoting large out of equilibrium deformations at discrete hotspots in lipid vesicles and thus inducing an overall membrane softening. Therefore, the rotation of ATP synthases promote mechanically adapted membranes with a high bending compliance and able to support high local curvatures. Our results evidence a mechanical functionality of the ATP synthase for biomembrane restructuring and shaping. Our results pave the way to new experimental realizations to explore the collective effects of rotating ATP synthases and their possible biological implications for biomembrane organization and protein functionality.

Actin cables, clouds, and comet tails organize mitochondrial networks in mitosis

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Symmetric cell division requires even partitioning of genetic information and cytoplasmic contents between daughter cells. While the mechanisms coordinating the replication and segregation of the genome have been extensively explored, the process by which organelles are apportioned remains less well-understood. Here, we identify multiple cytoplasmic actin assemblies that play distinct but convergent roles in mitochondrial organization in mitosis. First, we find a dense network of subcortical actin cables crisscrossing individual mitochondria in mitotic cells. This meshwork templates uniform 3D-mitochondrial positioning about the spindle and ensures the equal segregation of mitochondrial mass in cytokinesis. Second, we identify a CDC42/N-WASP/Arp2/3-dependent wave of actin filaments reversibly assembling on the surface of mitochondria to direct their motility. Within this wave mitochondria are either confined within branched-actin clouds or propelled by elongated, multi-tailed actin comets. Together, clouds and comet tails promote randomly oriented bursts of movement, which, over the duration of mitosis, effectively shuffles mitochondrial position in the mother cell to ensure their random and unbiased partitioning between daughter cells.
P1082

Two neuronal peptides encoded from a single transcript regulate mitochondrial function in *Drosophila*

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Naturally produced peptides (<100 amino acids) are important regulators of physiology, development, and metabolism. Recent studies have predicted that thousands of peptides may be translated from transcripts containing small open reading frames (smORFs). Here, we describe two previously uncharacterized peptides in *Drosophila* encoded by conserved smORFs, Sloth1 and Sloth2. These peptides are translated from the same bicistronic transcript and share sequence similarities, suggesting that they encode paralogs. We provide evidence that Sloth1/2 are highly expressed in neurons, localize to mitochondria, and form a complex. Double mutant analysis in animals and cell culture revealed that sloth1 and sloth2 are not functionally redundant, and their loss causes animal lethality, reduced neuronal function, impaired mitochondrial function, and neurodegeneration. These results suggest that phenotypic analysis of smORF genes in *Drosophila* can provide a wealth of information on the biological functions of this poorly characterized class of genes.

P1083

Mitochondrial Co-translational Protein Import and Network Structures Impact Mitochondrial Fragment Composition

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Mitochondria are hubs for metabolite and energy generation and have been shown to be very important for age-related processes, including cancer and neurodegeneration. Fragmented mitochondrial morphology is a hallmark of the dysfunction of mitochondrial activity and observed in those disease phenotypes. However, it remains unclear how eukaryotic cells coordinate protein production for those fragmented mitochondria. Mitochondrial proteins are mostly encoded in the nuclear genome and are imported from the cytoplasm to mitochondria. A fraction of mitochondrial protein-coding mRNA is localized to the mitochondrial outer membrane and co-translationally import proteins into the mitochondria, yet the impact of localized translation to these organelles on mitochondrial function is poorly understood. We made the novel observation that Tim50 protein, the translocase of the inner mitochondrial membrane and a co-translationally imported protein, is evenly distributed to mitochondria on average, however, the variation of the protein concentration was higher in mitochondrial fragments of the smaller size. We also observed the same phenotype in *fzo1Δ* mutant strains, in which the mitochondria showed fragmented morphology. To further test the involvement of translational regulation on this variability, we analyzed the mRNA movement on the mitochondrial outer membrane surface by utilizing the analysis of mitochondrial morphology through computational
modeling and the analysis of single molecule mRNA visualization using the MS2-MCP system. We observed that TIM50 mRNAs were trapped at single fragmented mitochondria in fzo1Δ mutant strains. This suggests that the co-translational protein import mechanism restricts mRNA movements to single mitochondrial fragments. We are currently testing whether the variation of the protein concentration in each mitochondrial fragment is based on the translation by changing the copy number of the mRNA. Our work shows that mitochondrial morphology affects mRNA localization and this could potentially result in heterogeneity of protein composition in each mitochondrial fragment. We propose this is a way to regulate the quality of mitochondrial fragments and accelerate the degradation of nonfunctional mitochondrial fragments.

New Techniques Using Fluorescence

P1084

Quantifying spatiotemporal dynamics of IRES and cap translation with single-molecule resolution in living cells
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Viruses use internal Ribosome Entry Sites (IRES) to hijack host ribosomes and promote cap-independent translation. While well studied in bulk, the dynamics of IRES-mediated translation remain unexplored at the single-molecule level. Here, we developed a bicistronic biosensor encoding distinct repeat epitopes in two open reading frames (ORFs), one translated from the 5'-cap, the other from the Encephalomyocarditis Virus IRES. When combined with a pair of complementary probes that bind the epitopes co-translationally, the biosensor lights up in different colors depending on which ORF is translated. Using the sensor together with single-molecule tracking and computational modeling, we measured the kinetics of cap-dependent versus IRES-mediated translation in living human cells. We show that bursts of IRES translation are shorter and rarer than bursts of cap translation, although the situation reverses upon stress. Collectively our data support a model for translational regulation primarily driven by transitions between translationally active and inactive RNA states.

P1085

Genetically Encoded Optical Sensors for Intra- and Subcellular pH Measurements
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Cancer cells display abnormal alkaline intracellular pH (pHi). Therapeutic approaches targeting intracellular alkalization therefore hold clinical promise, but these efforts have been hindered by tumor heterogeneity, highlighting an urgent need for tools to accurately map intratumoral pH. Furthermore, measuring the pH in subcellular compartments could improve the understanding of how pH is coupled to cancer cell metabolism and cellular processes. Genetically encoded tools, such as pH-responsive fluorescent proteins (FPs), have the potential to deliver these measurements. However, oxidizing intracellular conditions can cause FPs to misfold, aggregate, and become non-fluorescent. Here we
POSTER ABSTRACTS

describe the design, development, and testing of a new generation of genetically encoded, FP-based biosensors for the high-resolution visualization of intra- and subcellular pH. We have engineered robust, pH-responsive FPs capable of withstanding oxidizing intracellular conditions. To do so, DNA constructs for GFP-derived proteins were modified to be cysteine free and contain point mutations to promote protein folding, monomericity, and pH sensitivity. Proteins were expressed using inducible E. coli systems and purified via immobilized metal affinity chromatography. Photophysical properties and pH responsiveness were measured by fluorimetry in buffering solutions of incremental pH. Candidates that were bright and responded well to pH changes were assessed for their stability in the oxidizing bacterial periplasm, where fluorescence was evaluated using confocal laser scanning microscopy. We report five new spectrally distinct protein variants, ranging from cyan to yellow in color, that exhibit bright fluorescence, low cytotoxicity, and 5 to 15-fold changes in fluorescence intensity over pH 6.5-8.5 while in the bacterial periplasm. Moving forward, we plan to generate ratiometric biosensors with the ability to quantify pH, independently of local sensor concentration by fusing a pH-stable FP to each pH-responsive FP for signal normalization. Ultimately, we hope that cytoplasmic expressed sensors may be used to measure intratumoral pH, with a high degree of spatial localization, and that organelle targeted sensors may be used to examine cellular rates of glycolysis, oxidative phosphorylation, lipolysis, and other cancer-related metabolic pathways.

P1086

Emags, optimized vivid-derived magnets pair of photodimerizers for subcellular optogenetics
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Light-inducible dimerization modules enable precise temporal and spatial control of biological processes in non-invasive fashion. Among blue light-dependent dimerizers, Magnets are small (150 a.a.) protein modules engineered from the Neurospora crassa photoreceptor Vivid by engineering the homodimerization interface into complementary heterodimers. The two Magnets components do not homo-oligomerize and/or form aggregates upon light excitation, can be fused either at the N- or C-terminus of a protein or intracellular membrane-targeting localization sequences, and are functional when either or both components are tethered to intracellular membranes. As both components of the Magnets heterodimer are photoreceptors requiring simultaneous photoactivation to bind one another, this guarantees very low interaction in the dark and enables high spatiotemporal confinement of dimerization with a single excitation wavelength. However, existing Magnets require concatemerization for efficient responses and the expression of functional Magnets require an incubation of cells at 28°C, which limits their use for mammalian cells. Here we have overcome these limitations by engineering an optimized Magnets pair requiring neither concatemerization nor low temperature pre-incubation. We validated these “enhanced” Magnets (eMags) by using them to rapidly and reversibly recruit proteins to subcellular organelles, to induce/expand organelle contacts, and to reconstitute OSBP-VAP ER-Golgi tethering implicated in phosphatidylinositol-4-phosphate transport and metabolism. eMags represent a very effective tool to optogenetically manipulate physiological processes over whole cells or in small subcellular volumes.
Automated localization and quantification of RNA transcripts from RNA-FISH image data
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The ability to resolve the spatiotemporal localization of individual RNA molecules in single cells with high detail is important for studying the dynamics of gene regulation. RNA fluorescent in situ hybridization (RNA-FISH) is a frequently used technique for visualizing RNA in fixed cells using fluorescent probes. Automated processing of the resulting images is essential for large datasets that may include many different transcripts and timepoints. Here we demonstrate that our MATLAB based RNA-FISH image processing pipeline is a useful tool for automatically detecting the 3D locations of cell boundaries and RNA transcripts at single molecule resolution in an RNA-FISH image stack. In particular, this tool is effective for facilitating quantitative analyses of FISH data such as determining the colocalization of multiple transcripts or the relative amount of RNA in various subcellular compartments. Our spot detection approach using gaussian filtering and background image data for threshold calibration provides an additional software-based method of conducting batch FISH data analysis complementary to existing image processing tools.

Deep learning enables the identification and isolation of single cells of interest using high resolution images of non-labeled cells in flow

Classical cell classification and sorting techniques are limited by their reliance on pre-selected cell biomarkers or physical characteristics (e.g., size, density, electrical charge). Recent breakthroughs in machine intelligence and deep learning have achieved unprecedented accuracy across a wide range of image classification problems. We have developed a platform that combines a microfluidic apparatus, a high-resolution imager for unlabeled cells in flow, a Deep Neural Network (DNN) that enables the classification of cells based on their morphology, and a sorting mechanism to isolate rare cells of interest. The DNN classifier was trained on images of more than 25 million cells of multiple types captured on the platform. We built a model to discriminate among multiple cell classes, including fetal nucleated red blood cells (fNRBC), non-small-cell lung carcinomas (NSCLC), hepatocellular carcinomas (HCC), and blood cells. We then assessed model performance on a separate validation set of cell images, including cell lines not used in the training data. The results show that our classifier accurately discriminates among the classes modeled: for example, the classifier achieved an area under the ROC curve (AUC) metric of > 0.999 for the classification of NSCLC and hepatocellular carcinoma cell lines against a background of blood cells. We also demonstrate that features extracted from our classifier provide discriminating information on cell classes for which it has not been trained, suggesting that our model abstracts morphological attributes that are broadly informative of the type and state of cells. Finally, we demonstrate the enrichment of rare NSCLC cells in controlled spike-in cell mixtures. We accurately assess the purity of sorted cells by analyzing both common SNPs and known tumor-specific mutations in their DNA. We successfully isolated NSCLC cells from spike-in mixtures with WBCs or whole
blood at concentrations as low as 1:100,000, achieving an enrichment of > 25,000x on multiple cell lines. This work demonstrates that deep learning applied to high-resolution cell images collected at scale can achieve a high classification accuracy and can enable the label-free isolation of rare cells of interest for a wide range of applications.

P1089

**Genetically-encoded fluorescence-based reporters for measuring actin filament organization in living cells and tissues**

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Essential physiological functions, including cell division, cell adhesion and motility, and tissue morphogenesis, rely on the capacity of animal cells to change and adapt their shape. To accomplish these force-dependent tasks, animal cells make use of actin cytoskeletal filaments. The precise way in which actin filaments organize, i.e. how actin filaments are physically oriented in space, and how filament organization is remodeled in time, is determinant for force generation. Being able to measure actin filament organization directly in living cells and tissues thus promises to advance our understanding of how proteins and signaling pathways individually and collectively control actin-driven cellular functions. We will present the development of novel genetically-encoded, green- and red-fluorescent-protein-based reporters that allow non-invasive, quantitative measurements of actin filament organization in living cells and tissues by using polarization-resolved fluorescence microscopy. We will show examples of actin organization measurements in living mammalian cells in culture, as well as in living Drosophila and C.elegans embryos, and fission yeast cells.

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**High Resolution Mapping of Protein Concentrations in Living Cells**

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We are using several fluorescence microscope modalities to image mEGFP labeled structures in human induced pluripotent stem cells (hiPSC). To enhance the utility of this information, we have developed a method to quantify the intrinsic intensity information, creating image-based concentration maps. The method is applicable to several imaging platforms including confocal spinning disk, confocal point-scanning super-resolution, and lattice light-sheet microscopy. It is relatively simple, fast, and robust, using an EGFP dilution series, calibrated using fluorescence correlation spectroscopy (FCS), to convert fluorescence intensities into local concentrations. To validate the method, we used an hiPSC cell line expressing cytosolic mEGFP and measured its concentration, using point-FCS, as 70nM. Quantitative immuno-bLOTS using an average cell volume of 2x10⁻¹²l, determined from our imaging, yielded a concentration of 120nM. These values are in reasonable accord with the average concentration of 70nM determined by the presented method. We extended this method to estimate the protein concentration.
Most cell lines had one allele tagged with mEGFP. Immuno-blots revealed that for some cell lines the relative level of tagged to total protein was close to 50%, but others had lower values (e.g. nucleophosmin 22%, lamin B1 33%)\(^1\). We corrected the calibration curves accordingly. In addition, we used quantitative immuno-blots, on subcellular fractions, to estimate the fraction of labeled and unlabeled proteins incorporated into cellular structures, using this correction for molecules in organelles. We applied the method to seven mEGFP-tagged proteins from the Allen Cell Collection\(^2\). We estimated the total copy number of protein molecules per cell to be 0.08x10\(^6\) for PMP34 (peroxisomes), 0.3x10\(^6\) for Nup153 (nuclear pores), 0.5x10\(^6\) for Tom20 (mitochondria), 0.9x10\(^6\) for SERCA2 (sarcoplasmic reticulum/endoplasmic reticulum), 4x10\(^6\) for Sec61 beta (endoplasmic reticulum), 5x10\(^6\) for lamin B1 (nuclear envelope), and 23x10\(^6\) for nucleophosmin (nucleolus). Because these numbers were based on high-resolution, live cell images with sub-cellular resolution, we can quantify the relative amount and variations in structures correlated with location, environment and history. Finally, we showed that the method works with microscope data acquired with different microscope systems and settings. These data provide a spatially resolved estimate of protein concentration allowing study of the variance in protein concentrations in organelles and cell populations and also providing estimates of protein concentration for computational models of cell activities. \(^1\)Roberts, B., et al. (2017). Mol Biol Cell 28(21): 2854-2874. \(^2\)https://www.allencell.org/cell-catalog.html

P1091

**Optoproteomics enables image-guided subcellular protein identification**


Mapping the spatial proteome is essential to understand cellular activities underlying cell physiology and pathology. Current techniques lack the flexibility of capturing proteins in the subcellular region of interest (ROI) and consequently restrict scientists from resolving broad biology problems. Here, we introduce an integrated platform termed “optoproteomics”, which bridges the gap between optical imaging and mass spectrometry-based proteomics. High-throughput image acquisition and deep learning-based ROI assigning were applied to determine the desired area for patterned multiphoton illumination, which activated [Ru(bpy)]\(^{2+}\), a powerful photocatalyst, to drive tyrosine-specific labeling with a desthiobiotin-tyramide probe within few seconds per field. Moreover, peroxidase-assisted tyramide signal amplification was applied to fill tyrosine sites with probes at photo-labeled ROI for efficient enrichment of tagged-proteins. We showed that optoproteomics method can achieve high sensitivity and specificity on image-guided nuclear proteome identification, obtaining over a thousand of nuclear proteins, reaching 85% specificity, and detecting low abundance proteins at the copy number of ~1000. Furthermore, we expanded the applications of optoproteomics and performed imaged-guided precision labeling and protein identification of nucleoli, stress granules, and immune synapses. Together, our optoproteomics method is demonstrated to be a precise and robust approach that can be broadly applicable to diverse biology problems where spatial proteomes are on demand.
Nucelar Bodies and Phase Separation

P1092

Composition-dependent thermodynamics of intracellular phase separation
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Intracellular bodies such as nucleoli, Cajal bodies and various signaling assemblies represent membraneless organelles, or condensates, that form via liquid-liquid phase separation (LLPS). Biomolecular interactions—particularly homotypic interactions mediated by self-associating intrinsically disordered protein regions—are thought to underlie the thermodynamic driving forces for LLPS, forming condensates that can facilitate the assembly and processing of biochemically active complexes, such as ribosomal subunits within the nucleolus. Simplified model systems have led to the concept that a single fixed saturation concentration is a defining feature of endogenous LLPS, and has been suggested as a mechanism for intracellular concentration buffering. However, the assumption of a fixed saturation concentration remains largely untested within living cells, in which the richly multicomponent nature of condensates could complicate this simple picture. Here we show that heterotypic multicomponent interactions dominate endogenous LLPS, and give rise to nucleoli and other condensates that do not exhibit a fixed saturation concentration. As the concentration of individual components is varied, their partition coefficients change in a manner that can be used to determine the thermodynamic free energies that underlie LLPS. We find that heterotypic interactions among protein and RNA components stabilize various archetypal intracellular condensates—including the nucleolus, Cajal bodies, stress granules and P-bodies—implying that the composition of condensates is finely tuned by the thermodynamics of the underlying biomolecular interaction network. In the context of RNA-processing condensates such as the nucleolus, this manifests in the selective exclusion of fully assembled ribonucleoprotein complexes, providing a thermodynamic basis for vectorial ribosomal RNA flux out of the nucleolus. This methodology is conceptually straightforward and readily implemented, and can be broadly used to extract thermodynamic parameters from microscopy images. These approaches pave the way for a deeper understanding of the thermodynamics of multicomponent intracellular phase behavior and its interplay with the nonequilibrium activity that is characteristic of endogenous condensates.

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An Intrinsic Disordered Region Mediated Confinement State Contributes to Dynamics and Function of Transcription Factors
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Transcription factors (TFs) regulate gene expression by binding to specific consensus motifs within the local chromatin context. The mechanisms by which TFs navigate the nuclear environment as they search for binding sites remains unclear. Here, we used single-molecule tracking and machine-learning based classification to directly measure the nuclear mobility of the glucocorticoid receptor (GR) in live cells. We revealed two distinct and dynamic low-mobility populations. One accounts for specific binding to chromatin, while the other represents a confinement state that requires an intrinsically disordered region (IDR), implicated in liquid-liquid condensate subdomains. Further analysis showed that the dwell times of both subpopulations follow a power-law distribution, consistent with a broad distribution of affinities on the GR cistrome and interactome. Altogether, our data link IDRs with a confinement state that is functionally distinct from specific chromatin binding and modulates the transcriptional output by increasing the local concentration of TFs at specific sites.

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Liquid-Liquid Phase Separation Drives Compartmentalization and Protection of Telomeres

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Chromatin, a nucleic acid and protein-based polymer, is dynamically organized into spatially and functionally distinct compartments. Given chromatin’s polymeric nature, the 3D architecture of the genome may be compartmentalized by liquid-liquid phase separation (LLPS). Telomeres are a particularly interesting genomic compartment comprised of kilobases of repetitive DNA and a six-protein complex named shelterin that work together as a unit to protect the single-stranded ends of chromosomes from aberrant recognition by DNA damage repair proteins. Here, we tested the hypothesis that telomere organization is driven by LLPS. However, because telomeres rarely encounter one another in living cells due to their constrained subdiffusive motion, it is difficult to test in vivo whether two telomeres can coalesce, a key aspect of the liquid condensate model. To overcome this challenge, we use a novel optogenetic approach that brings two telomeres into close proximity and find that telomeres readily coalesce into and remain as a single liquid-like droplet. We further characterize the biophysical phase behavior of telomeres, which exhibit signatures of multicomponent condensates, wherein both homotypic and heterotypic interactions between the shelterin proteins and the scaffolding telomeric DNA build the compartment. These findings are consistent with in vitro experiments, which reveal that shelterin complex proteins readily phase separate together with telomeric DNA. This work not only develops a better understanding of the biophysical mechanism of the end-protection mechanism, but it also elucidates general principles underlying the structure and composition of nuclear compartments.
P1095

**The oncogenic fusion protein FUS-CHOP undergoes nuclear liquid-liquid phase separation**

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Myxoid liposarcoma is caused by a chromosomal translocation resulting in a fusion protein comprised of the N-terminus of FUS (fused in sarcoma) and full-length CHOP (CCAAT/Enhancer Binding Protein Homologous Protein). FUS is an RNA binding protein that functions in RNA metabolism and CHOP is a stress-induced transcription factor. In the diseased state, the FUS-CHOP fusion protein is an atypical transcription factor that causes unique gene expression and oncogenic transformation. The mechanism of FUS-CHOP-induced transcriptional activation is unknown. Recently, RNA polymerase, the FET family proteins, some transcription factors, and super enhancers were shown to undergo liquid-liquid phase separation, forming membraneless compartments at transcriptional start sites, to control mRNA synthesis. The N-terminus of FUS is both necessary and sufficient for FUS phase transition, therefore, we hypothesized that the aberrant transcriptional activation by FUS-CHOP is the result of the N-terminus of FUS driving FUS-CHOP phase transition in the nucleus. We characterized ectopically expressed FUS-CHOP nuclear puncta using live cell imaging and confirmed that FUS-CHOP is phase separating. Further, we show this phase separation is dependent on the N-terminus of FUS. We propose that FUS-CHOP phase transition is a novel mechanism and therapeutic target for treatment of myxoid liposarcoma.

P1096

**Mechanisms of phase separation-regulated biosynthetic activity of nucleolus**

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The nucleolus is a membrane-less, protein/RNA enriched organelle that produces ribosomes. Nucleoli are spherical, dynamics and limited in number in healthy cells, but changes in their size, shape and number have been implicated in malignant growth, aging and metabolic disorder in mammalian cells. The nucleolus consists of three sub-compartmental: the fibrillar center (FC), the dense fibrillar component (DFC), and the granular component (GC), where pre-ribosomal RNA transcription, rRNA processing, and assembly of ribosomal subunits take place, respectively. The nucleolus is sensitive to various cues (e.g., developmental signals and nutrition). Recent work indicates that liquid-liquid phase separation (LLPS) is responsible for the formation of the nucleolus. The liquid-like nature of nucleoli is thought to facilitate ribosome biogenesis. However, the molecular mechanism of how the LLPS of the nucleolus correlates with its biological function remains largely elusive. In this project we examined the nucleolus at different developmental stages of *C. elegans* by fluorescently labelling marker proteins for each sub-compartment. We observed that intestinal nucleoli increase in size and tend to become less spherical through larval development. These preliminary results suggest that the nucleolus may undergo a liquid-to-solid transition during development, which may reduce the efficiency or accuracy of ribosome biogenesis.
Polarity in Development

P1097

Polarity Establishment in the Developing Zebrafish Epidermis is Regulated by aPKC and E-cadherin

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The epidermis is a multi-layered epithelium that covers the vertebrate body. It is developed from a bilayered epithelium, the outer periderm and inner basal epidermis. This epithelium is polarized along the apicobasal axis at the tissue level. However, how this polarity is established in the developing epidermis remains underexplored. In simple epithelia, the apicobasal polarity is regulated by polarity pathways such as the apical aPKC-Par3-Par6 and Crb-Sdt-PatJ pathways, and basolateral Lgl-Dlg-Scrib and Yurt-Cora-NrxIV-ATPase pathways. Epithelial cells possess various cell-cell junctions—adherens junctions, desmosomes, and tight junctions, to maintain epithelial integrity. In simple epithelia, the adherens junctions are apically polarized and the polarity pathways regulate the formation, and positioning of the adherens junctions. Similarly, perturbing the adherens junctions perturbs the localization of the polarity regulators. In multi-layered epithelia, the distribution of adherens junctions and their interactions with the polarity pathways remain unknown. We use the developing zebrafish bilayered epidermis to answer these questions. We show a polarized localization of the adherens junctions in the developing zebrafish epidermis. The levels of adherens junctions increase along the apicobasal axis in the periderm and decrease in the basal epidermis, with the maximum levels at the interface of the two layers. Our data reveals that aPKC regulates the robustness of the polarized distribution of E-cadherin, a key adherens junction component, in the periderm. This loss of robustness is independent of levels of E-cadherin or cell morphology. We demonstrate that levels of E-cadherin in one layer regulate the levels of E-cadherin in the juxtaposing layer in a layer non-autonomous manner. Additionally, E-cadherin is critical in transducing polarity cues from one layer to another. Our data show that perturbing E-cadherin in one layer, disrupts localization and levels of Lgl in a layer autonomous and layer non-autonomous manner. With these data, we propose a model of stepwise polarization of the developing zebrafish epidermis. During development, periderm forms first from the enveloping layer, wherein aPKC initiates the polarization of E-cadherin. The polarity cues from periderm are transduced from periderm to the basal epidermis via E-cadherin. E-cadherin in the basal epidermis then regulates Lgl levels and hemidesmosome formation, thus, polarizing the bilayered epidermis.

P1098

A Purse-string Mechanism ensures Convergence of the Anteroposterior axis to Embryo shape in Early C. elegans development

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Proper establishment of body axes is an essential step in development. It has been observed in many organisms that the body axes align with geometric features of the surroundings in which the embryos develop. However, the mechanisms that underlie the alignment of the body axes to these geometric
features are poorly understood. We focus on the alignment between body axes and geometry by investigating the Anteroposterior (AP) axis establishment in *Caenorhabditis elegans* embryo. In the one-cell *C. elegans* embryo, the AP axis is encoded in the orientation of the partitioning-defective (PAR) cell polarity domains. **These PAR domains align along the geometric long axis of the ellipsoidal embryo** (a shape constraint imposed by the rigid eggshell); **however, the mechanism behind this alignment remains poorly understood.** Here we show that the mechanical forces, which generate flows in the actomyosin cortex during PAR domain establishment, are essential for PAR domain alignment. By combining quantitative data with predictions from theoretical modeling and genetic perturbations, we show that the primary mechanism behind the alignment is a self-organised line-tension in a nematic actomyosin cortex. This line-tension manifests morphologically as a partial ingression - the so-called pseudocleavage furrow. We also use genetic perturbations to modify the pseudocleavage mechanics, allowing us to re-programming the alignment process to a state where it promotes misalignment of the PAR domains. In addition, we show that the cytoplasmic streaming induced by the actomyosin flows serve a minor secondary role in the alignment process, albeit with very reduced dynamics of the PAR domain alignment. Together, **these results show that mechanics of the nematic actomyosin cortex drive the alignment of the PAR polarity domains, and thus the AP axis of the embryo, along the geometric long axis of the ellipsoidal embryo.** We thus show that mechanical forces are crucial for the geometric sensitivity and proper establishment of the AP axis in the early *C. elegans.*

**P1099**

**Cell polarity determinant Dlg1 facilitates epithelial invagination by regulating tissue-scale mechanical coordination**  
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Apical constriction mediated epithelial folding serves as a fundamental mechanism to convert epithelial sheets into multilayered tissues. In *Drosophila* gastrulation, ventrally localized prospective mesoderm cells constrict apically and subsequently invaginate to form a ventral furrow (VF). While the role of apical constriction in VF formation has been well demonstrated, it remains elusive whether additional molecular and mechanical inputs, other than apical constriction, play any role in folding. We found that depletion of the apical-basal polarity determinant, Dlg1, results in a delay in VF invagination and a reduction in invagination depth without affecting the rate of apical constriction. The invagination defect is associated with an altered behavior in the neighboring non-constricting cells that is detectable soon after the onset of apical constriction. In the *dlg1* RNAi mutant, non-constricting cells exhibit an irregular, overextended apical morphology when they are pulled by the constricting cells, and this seems to slow down the overall tissue movement towards the ventral midline. Using an *in vivo* magnetic tweezers-based approach, we found that the mutant tissue is less elastic and more prone to irreversible deformation than wild type. The impaired mechanical properties of *dlg1* RNAi embryos are associated with a basal dispersion of the normally subapical actin cytoskeleton in the non-constricting cells. To directly test the role of non-constricting cells in facilitating VF ingression, we disrupted the non-constricting cells by performing spatially confined laser microdissection or optogenetic depletion of cortical actin. Both treatments impaired VF ingression. Our findings demonstrate that robust VF formation requires tissue-level cooperation between constricting and non-constricting cells, which is contingent on the mechanical integrity of the non-constricting cells.
Investigating oriented divisions in the early Drosophila embryo

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Investigating oriented divisions in the early Drosophila embryo J. M. Camuglia, A. C. Martin

Epithelial morphogenesis and homeostasis are properly achieved by oriented cell divisions that occur along specific directions relative to both the epithelial plane and the polarity of an organ/organism. In epithelial sheets of cells, divisions can occur either perpendicular or parallel (planar) to epithelial sheets. Planar cell divisions can further exhibit polarity in specific orientations along epithelial tissues (planar polarization). Spindle orientation is often achieved by a complex of Pins/LGN, Mud/NuMa, Gαi, and Dynein, which interacts with astral microtubules to rotate the spindle. Here, we identify the mitotic domains of the early embryo as a system to study Pins-mediated oriented divisions during morphogenesis. Mitotic domains, 1, 3, and 5, but not 2, exhibit oriented division and these oriented divisions are dependent on proper Pins localization and activity. We find that the divisions within these domains are oriented within 30 degrees of the anterior-posterior (AP) axis of the embryo and that Pins is localized in planar polarized crescents within the domains. Disruption of Pins localization and activity via expression of a myristoylated version of Pins leads to misoriented divisions. It is currently unknown what causes Pins localization in this context. Both planar polarity proteins and mechanical force have been shown to localize Pins and cue spindle orientation in planar divisions. We find that in mitotic domains 1, 3, and 5, canonical planar cell polarity pathways are not solely responsible for the orientation of the divisions. Further, we find that components of adherens junctions, which mechanically couple cells within epithelia, are necessary for the orientation of the divisions. Disruption of α-catenin, β-catenin, and p120-catenin results in divisions that fail to orient along the AP axis. Finally, we propose that the oriented, planar divisions in the head region of the early Drosophila embryo function to push the tissue causing an elongation in the direction of division.

P1101

Spatiotemporal Markers of the Epithelial-Mesenchymal Transition During Sea Urchin Gastrulation

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Spatiotemporal Markers of the Epithelial-Mesenchymal Transition During Sea Urchin Gastrulation

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Cell motility is a central feature of animal development and a critical factor contributing to many pathologies. In the sea urchin embryo, Primary Mesenchyme Cells (PMCs) undergo an Epithelial Mesenchymal Transition (EMT), cross a basement membrane and enter the blastocoel, where they undergo a final division prior to terminal differentiation. And while the gene regulatory network for PMC specification and EMT has been characterized in detail, the actual conditions and subcellular alterations that result in polarity reversal and the initiation of motility remain poorly understood. In an effort to determine the role of cell cycle in regulating EMT in sea urchin embryos, we expressed a nuclear targeted CDK2 biosensor that is exported from the nucleus as cells progress through S and G2

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phases. Measurement of the cytoplasmic: nuclear ratios of the biosensor revealed that cells undergoing EMT had significantly higher nuclear biosensor levels than cells following ingestion, and synchronization of embryos in G1, S or G2 revealed that while G1 arrest had no effect on EMT, arrest in S or G2 inhibited ingestion. Together, these results suggest that the timing of EMT is linked to the cell cycle. To better understand the morphological transitions that occur during polarity reversal and EMT, we examined the apical polarity marker, Par-6. Par-6 is recruited to the membrane following fertilization and is polarized as early as the 2 cell embryo. Prior to PMC ingestion, Par-6 was internalized into intracellular compartments that bear a strong resemblance to liquid-liquid phase separations (LLPs). And while depletion of transcription factors known to drive EMT (Snail, Tbr & Foxn2/3) significantly depressed PMC ingestion, there was only a mild effect on Par6 internalization. Together, these results suggest the existence of novel cellular conditions that must be met before cells can undergo EMT, and current efforts are focused on understanding interrelationship between the cell cycle, polarity reversal, and the gene regulatory networks that ultimately drive PMC ingestion.

P1102

**E-cadherin-mediated cell contacts underlie epithelial symmetry breaking in vivo**

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Epithelia provide resiliency to organs and act as environmental barriers, functions that depend on establishing polarized apical surfaces facing the exterior and basolateral surfaces oriented toward the body. Achieving this polarity requires symmetry breaking, the process by which apico-basolateral axis orientation is initially defined. Epithelial symmetry breaking must occur at both the cellular level (every cell adopts polarity) and at the tissue level (cells align their individual polarity axes to generate a polarized epithelium). While critical for tissue function, the molecular signals underlying epithelial symmetry breaking at these two scales are poorly understood. To determine such mechanisms we use the *C. elegans* embryonic intestine, a simple epithelium where cell and tissue symmetry breaking are temporally separable. Apical proteins initially coalesce as puncta at intestinal (E) cell membranes before undergoing a tissue-wide migration to the intestinal midline to define the apical surface. We find that these puncta localize to E/E homotypic contacts and are excluded from contacts between E and non-E neighbors, thus we hypothesized that symmetry breaking is informed by tissue-wide contact asymmetry created by the stereotypic arrangement of intestinal precursors. Consistently, altering this arrangement using mutants with ectopic E/E contacts is sufficient to change apical protein localization. To explore modes of E/E contact recognition, we tested a role for the adhesion protein E-cadherin/HMR-1, which we found localized at homotypic puncta ahead of apical proteins. Through intestine-specific protein depletion (HMR-1<sup>gut(−)</sup>), we show that HMR-1 is required for cellular symmetry breaking as apical puncta formation is significantly delayed in HMR-1<sup>gut(−)</sup> embryos. HMR-1 further plays a role in tissue symmetry breaking as the apical puncta that eventually form are misoriented and fail to migrate to the midline. Surprisingly, these defects ultimately correct as mislocalized puncta are swept to the midline through an apparent HMR-1-independent mechanism, resulting in severely delayed but viable animals. These data support a model in which E-cadherin contributes to: 1) cellular symmetry breaking by designating homotypic contacts and creating a platform for apical proteins at the onset of polarization; and 2) tissue symmetry breaking by guiding apical proteins to the tissue midline while also highlighting a role for redundant mechanisms in this process. As internal epithelia must interpret their environment to break symmetry despite being surrounded by other cells, this model offers a logical framework for how such
contact asymmetries can inform polarization through an E-cadherin based mechanism that likely plays a similar role in other tissues.

P1103

**The PAR polarity complex promotes epithelial integrity during intestinal morphogenesis**

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Epithelial tissues are comprised of adherent polarized cells that line organs and create selective barriers to the outside world. To do this, epithelial cells polarize along an apicobasal axis; apical surfaces face the lumen, and neighbors adhere via junctional complexes to form a continuous surface. Yet during development and homeostasis, epithelia face assaults on their integrity such as cell division, shape change, and physical forces. The developing *C. elegans* intestine provides an excellent *in vivo* epithelial model to study how epithelia overcome these assaults while maintaining barrier function and apical continuity. The intestinal primordium consists of 16 polarized cells with apical surfaces facing a central midline, the future lumen. Four cells divide following polarization, and the resulting 20 cells elongate and build an intestinal tube. Using live imaging, tissue-specific protein depletion, and genetic screening approaches, we are investigating how this polarized epithelium maintains apical continuity through mitosis and tissue elongation *in vivo*. To understand what happens to polarized features during mitosis, we live imaged cytoskeletal, polarity, and junctional proteins during intestinal cell divisions and observed that ACT-5/actin, apical PAR proteins, and the junctional protein HMR-1/E-cadherin remain localized during mitosis. In contrast, apical microtubules and their associated proteins are transiently lost during mitosis and returned to the apical surface after mitosis completes. This loss of apical microtubules appears coupled to the building of the microtubule-based mitotic spindle, suggesting a functional switch between these structures. Based on our localization findings, we hypothesized that PAR proteins act as a memory mark, directing the return of apical microtubules after mitosis. Using intestine-specific depletion of PAR-6/Par6 and PKC-3/aPKC, we found that the return of microtubules to the apical surface is defective following mitosis, supporting our hypothesis. In addition, we found that intestine-specific depletion of PKC-3 or PAR-6 causes apical surfaces and junction proteins between neighboring cells to become discontinuous during elongation. The consequence of this discontinuity is larvae that die with edematous intestines that are unable to pass food. These experiments reveal a role for PAR proteins in maintaining epithelial integrity through mitosis and elongation, two critical steps in epithelial development and function across organisms. Funding: NIH K99GM13548901

P1104

**Frizzled3 restricts the formation of the anterior PCP complex in the vertebrate neural plate through Vangl2 phosphorylation**

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Planar cell polarity (PCP) refers to the orientation of cells in the plane of the tissue. PCP is thought to be controlled by the feedback between the oppositely localized Vangl-Prickle and Frizzled-Dishevelled protein complexes, but experimental evidence for this model *in vivo* is limited. In the *Xenopus* neural plate, a complex of Vangl2 with Prickle3 accumulates at the anterior cell boundaries, but whether this protein association is regulated by other PCP proteins has been unknown. Here we apply biochemical
approaches to study proteins interactions in the embryo to show that overexpression of Frizzled3, one of the core PCP components, reduced the interaction between Vangl2 and Prickle3. Conversely, Vangl2-Prickle3 interaction was enhanced in Frizzled3-depleted embryos, supporting the view that Frizzled3 is a negative regulator of the Vangl-Prickle association. We also identified the amino acid residues that become phosphorylated in response to Frizzled3. Compared to the wild type Vangl2, the association of phosphomimicking Vangl2 mutant with Prickle3 was weaker, suggesting the negative role of this phosphorylation in the PCP complex formation. Importantly, the effect of Frizzled3 on the interaction and colocalization of Prickle3 with the phosphomutant Vangl2 was strongly reduced, confirming our hypothesis. We also found that the anterior localization of the phosphomutant Vangl2 in the neural plate was reduced confirming the role of Vangl2 phosphorylation in PCP. Our data provide the first evidence for the in vivo regulation of the Vangl2-Prickle3 interaction by a Frizzled receptor and identify a specific Vangl2 phosphorylation event as an underlying mechanism.

P1105

Self-organising polarisation of migrating cells in vivo.
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Cell migration plays a fundamental role in many key biological processes both in physiological and pathological conditions. In order to be able to migrate, cells need to establish front that pushes forward and a rear that retracts. In the case of bleb-driven migrating cells, the mechanisms regulating their polarization are not completely understood. In this work, we used primordial germ cells (PGCs) of the zebrafish as a model to study spontaneous polarization of blebbing cells migrating in vivo. We determined the molecular and morphological cascade that leads to cell polarization and the cross dependency among the different polarized elements identified. We show how filamentous actin at the cell front recruits the myosin light chain kinase and promotes the formation of membrane invaginations favouring blebbing at the cell front. At the same time, we identified a role for these polymerized actin structures as a biophysical barrier that facilitates front bleb expansion while controlling their composition. Furthermore, actin mediated retrograde flow promotes the transport of proteins with bleb inhibiting characteristics to the cell rear, generating a robust front-rear polarity in these cells.

Synaptic Signal Transmission

P1106

Activity-Dependent-Bulk Endocytosis is hijacked to dismantle presynaptic terminals in remodeling neurons
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Across phylogeny, presynaptic terminals are highly dynamic during development, but the underlying cellular mechanisms remain elusive. To address this question, we investigate synaptic remodeling in the
**C. elegans** motor circuit in which axo-dendritic compartments repolarize as the presynaptic boutons of Dorsal D (DD) neurons are moved to new locations during early larval development. Our findings suggest that the presynaptic ENaC channel, UNC-8, triggers a Ca\(^{++}\)-dependent mechanism similar to Activity-Dependent Bulk Endocytosis (ADBE) to dismantle presynaptic components for reassembly at nascent synapses. ADBE functions in highly active neurons to recycle synaptic vesicle membrane. In ADBE, elevated Ca\(^{++}\) activates the phosphatase, Calcineurin, which targets dynamin and other "dephosphins" to drive an endocytic mechanism involving the F-BAR protein, syndapin, and branched-actin polymerization. We have confirmed that DD synaptic remodeling also depends on intracellular Ca\(^{++}\), involves presynaptically-localized Calcineurin, requires dynamin and other dephosphins, is mediated by syndapin and depends on Arp2/3-driven actin polymerization. Live-cell imaging experiments have revealed that actin dynamics is elevated during DD remodeling and instances in which actin polymerization precedes the removal of synaptic components. Notably, EM reconstruction at remodeling presynaptic terminals detect large (70-100nm), clear spherical structures resembling bulk endosomes. Thus, our findings suggest that ADBE, which normally functions to maintain neurotransmitter release at local synapses has been effectively repurposed to dismantle presynaptic terminals for reassembly at new locations. Additional findings have provided a novel explanation for this unanticipated phenomenon. An earlier report showed that the synaptic vesicle protein, RAB-3, is recycled for use at nascent synapses in remodeling DD neurons. We have confirmed this observation and shown that the Arp2/3 activator, TOCA-1, is required for RAB-3 recycling to new DD synapses. This finding is intriguing because the Arp2/3 activators, TOCA-1 and the Wave Regulatory Complex, which promote DD remodeling, have also been shown to mediate recycling of membrane proteins in *C. elegans* intestinal cells. Consistent with this model, the recycling endosome GTPase, RAB-11, functions in DD neurons to eliminate synaptic vesicle proteins. Thus, our findings support a model in which an ADBE-like mechanism mediates the removal of presynaptic components for delivery to new synapses. This work is important because it describes a cellular mechanism involving conserved proteins that could function in the developing brain when synaptic boutons are highly dynamic.

P1107

**Effect of Homeostatic scaling on the nanoscale organization of presynaptic proteins like Voltage Gated Calcium Channels and active zone marker Bassoon**

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**Title: Effect of Homeostatic scaling on the nanoscale organization of presynaptic proteins like voltage gated calcium channels and active zone marker Bassoon Abstract:** Learning and memory are some of the brain’s most remarkable features which are believed to emerge from elementary properties of chemical synapses. Evoked release happens when the action potential invades the presynaptic bouton which leads to the exocytosis of synaptic vesicle encapsulating the neurotransmitter. Previous studies have showed how neurons employ either presynaptic or postsynaptic mechanisms to counteract activity blockade depending on the age of the synapse. In this project we chose to study two different presynaptic proteins important for structural and functional integrity of a synapse which are Bassoon and Voltage gated Calcium Channels (VGCC) and the effect of homeostatic scaling on their spatial organization. Using super resolution microscopy techniques like STED, our results showed that homeostatic scaling causes differential nanoscale spatial distribution of these presynaptic proteins in...
young and mature synapses. In conclusion, homeostatic scaling alters the organization of these presynaptic proteins depending on the developmental stage of a synapse.

P1108

**Action potential-coupled Rho GTPase signaling drives presynaptic plasticity**  
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Proper nervous system function requires dynamic remodeling of the synaptic actin cytoskeleton. Although actin dynamics and regulation have been well-characterized in postsynaptic spines, the presynaptic cytoskeletal processes engaged during the synaptic vesicle cycle remain poorly resolved. To identify these signaling pathways, we conduct a proximity-based proteomics analysis of presynaptic terminals using *in vivo* biotin identification (BioID). The resultant proteome is heavily enriched for actin cytoskeleton regulators, including Rac1, a Rho GTPase that activates the Arp2/3 complex to nucleate branched actin filaments. Surprisingly, we find that Rac1 and Arp2/3 are closely associated with presynaptic vesicle membranes, and their presynaptic depletion affects synaptic vesicle replenishment at both excitatory and inhibitory synapses. Using new optogenetic tools and fluorescence lifetime imaging approaches, we show this pathway sculpts short-term presynaptic plasticity and that its activation is coupled to action potentials by voltage-gated calcium influx. Thus, this study provides a new proteomic framework for understanding the presynaptic cytoskeleton, and uncovers a new presynaptic actin signaling pathway that is conserved across cell types. Additionally, it calls into question the current view that Rho GTPase signaling and its involvement in neurodevelopmental disorders is largely a postsynaptic phenomenon.

P1109

**Cyclic AMP controls a trafficking mechanism that directs the neuron specificity and subcellular placement of electrical synapses**  
**S. D. Palumbos**, R. L. Skelton, C. E. Strothman, R. McWhirter, M. Zanic, D. M. Miller, III; Vanderbilt University, Nashville, TN.

Despite the functional importance of electrical synapses, the molecular mechanisms that direct the formation of neuron-specific gap junctions remain largely unknown. To address this question, we identified targets of the UNC-4 transcription factor that controls connectivity in the *C. elegans* motor circuit. UNC-4 functions in VA motor neurons to direct the formation of gap junctions on the VA axon with the interneuron AVA (VA-AVA). Locomotion is disrupted in *unc-4* mutants because VAs are miswired with electrical input from the interneuron AVB (VA-AVB) which aberrantly form on the VA soma. Thus, UNC-4 controls both the specificity and subcellular placement of electrical synapses. We determined that UNC-4 blocks expression of the phosphodiesterase, PDE-1, to prevent assembly of ectopic VA-AVB gap junctions. This finding suggests that cAMP signaling promotes the formation of
functional wild-type VA-AVA gap junctions. We validated this hypothesis by showing that optogenetic elevation of cAMP rescues the Unc-4 movement defect and thus restores VA-AVA circuit function. In addition, forced depletion of cAMP in VAs phenocopies unc-4 mutants. Because gap junction placement is shifted from the VA axon to cell soma in unc-4 mutants, we reasoned that trafficking of gap junction components could be perturbed. Live-cell imaging of the gap junction protein, GFP-UNC-9, confirmed that trafficking into the VA axon is strikingly impaired in unc-4 mutants. Genetic activation of cAMP signaling is sufficient to restore GFP-UNC-9 trafficking in VAs. Thus, we propose that cAMP directs both the specificity and placement of electrical synapses by activating mechanisms that transport gap junction components into the VA axonal compartment. We determined that an additional UNC-4 target, the atypical kinesin VAB-8, is also ectopically expressed in unc-4 mutant VAs where it blocks GFP-UNC-9 trafficking. We hypothesize that cAMP antagonizes VAB-8-dependent disruption of gap junction trafficking to maintain functional VA-AVA electrical synapses. These findings are important because previous studies in cultured cells have implicated cAMP signaling in gap junction assembly, but this mechanism has not been previously investigated in an intact nervous system.

P1110

Coordination of synaptic vesicle acidification and trafficking by Rabconnectin-3a
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Vesicle acidification is essential for many cellular processes, including synaptic vesicle (SV) refilling and neurotransmission. Despite its critical importance, the regulation of the vacuolar H+-ATPase (v-ATPase) activity on SVs remains poorly understood. It is also unclear how impaired acidification affects subsequent steps in SV recycling. Here, we show that Rabconnectin-3a, a largely uncharacterized 340-kDa essential protein linked to neurodevelopment, mental retardation, hearing loss and polyendocrine-polyneuropathy syndromes, stabilizes the v-ATPase expression and activity in mammalian cells, and is present at multiple SV recycling intermediates. Neurons without Rabconnectin-3a have impaired neurotransmission and altered SV recycling: their SVs do not acidify efficiently and fail to recruit Rab3, a marker of mature SVs that controls SV trafficking. Inhibition of vesicle acidification per se affected Rab3 recruitment similarly to Rbcn-3a ablation. Our data reveal a tight coordination between vesicle acidification and trafficking, with Rabconnectin-3a acting as a scaffold that links machineries for exocytosis, acidification and (re)filling.

P1111

Role of AP3 Complex in Dopamine Release
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In contrast to other neurotransmitters, dopamine is released from dendrites as well as axons. However, the regulation and relative role of dopamine release from these two sites remains poorly understood. I have focused on the vesicular monoamine transporter VMAT2 because it defines the vesicles capable of exocytic monoamine release. We have recently found that the heterotetrametric adaptor protein AP-3 complex has a specific role in the trafficking of VMAT2, not the vesicular transporters for other classical transmitters (Silm et al., 2019). In particular, loss of AP-3 impairs the recycling of synaptic vesicles expressing VMAT2. In addition, AP-3 deficiency reduces the targeting of VMAT2 to the axon terminal. In this study we have shown that loss of AP-3 reduces the axonal storage of dopamine and remarkably, increases storage in midbrain dendrites. The AP-3 complex occurs in both ubiquitous and neuronal isoforms. To determine whether local recycling and axonal polarity of VMAT2 depend on different isoforms of AP-3, we used knockout mice specific for either the ubiquitous or neural isoform of the beta3 subunit. The ubiquitous beta3A knockout shows no effect on dopamine storage in either the striatum (axon) or midbrain (dendrites). In contrast, loss of the neuronal beta3B reduces the striatal storage of dopamine very similar to the full AP-3 knockout. At the same time, loss of AP-3 (total or the neuronal isoform) increases midbrain dopamine, indicating redistribution of VMAT2 to the dendrites. We also find that the neuronal AP-3 isoform localizes preferentially to the axon. Indeed, loss of beta3B but not beta3A impairs presynaptic recycling of VMAT2, with no effect on a vesicular glutamate transporter. In addition, loss of the neuronal isoform reduces axonal dopamine release observed by fast scanning cyclic voltammetry. Further, we find that loss of the neuronal isoform specifically impairs the response to high frequency stimulation, with much less effect at low frequency. Thus, the neuronal isoform of AP-3 targets VMAT2 specifically to a population of low release probability vesicles. We hypothesize that this is required to sustain dopamine release in response to burst firing, which mediates the role of dopamine neurons in reinforcement learning.

P1112

**Measuring and Standardization of BDNF in heparin-plasma blood samples using a commercial ELISA: pitfalls and solutions**

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**Introduction:** Brain-Derived Neurotrophic Factor (BDNF) is a neurotrophin that plays an essential role in neuronal development and synaptic plasticity. Enzyme-linked immunosorbent assay (ELISA) kits can be used to measure BDNF in blood serum. However, information about sample processing and appropriate standards when clotting factors are present in the sample remains scattered. We aim at validating and optimizing the BDNF ELISA kit with respect to measurements in heparin-plasma blood samples.

**Methods:** Blood heparin-treated plasma from 28 adults were processed as the commercial kit Mature BDNF/proBDNF Combo Rapid ELISA Kit (BEK-2211/2237, Biosensis, Australia) suggested for human plasma. **Results:** we found that the pre-analytical conditions were critical for plasma samples. Determination of the intra assay variation and the accuracy and yield of the BDNF ELISA kit in heparin-plasma samples were conducted with the optimal dilutions. A linear dilution curve determined the optimal dilutions of plasma extracts; we found that 1:2 dilution optimized the detection levels for proBDNF and 1:50 dilution for matureBDNF. The range of sensitivity for BDNF isoforms in heparin-plasma samples was accurate to the manufacturer’s descriptions. Furthermore, we established the
coefficient of variation (CV%) between pro- and mature BDNF. The inter-assay variation showed a CV% of 5% for mature BDNF and 25% for proBDNF. We conclude that the BDNF ELISA kit determines heparin-plasma BDNF accurately and with high reproducibility. Last, it can be used for the measurement of BDNF isoforms in samples with clotting agents. Comparisons between clotting-plasma and serum samples will be determined and presented as part of this pilot study.

Tumor Invasion and Metastasis: Mesenchymal Transition

P1113

*Drosophila Yki*<sup>35/A</sup> midgut tumor cell dissemination as an *in vivo* model of mesenchymal cell migration

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The spread and colonization of tumor cells at distant sites, refer to as metastasis, is strongly correlated to the poor prognosis of cancer patients. Due to its high impact on cancer mortality, numerous mammalian studies often focus on inhibiting the formation of distant colonies to prevent metastasis. Meanwhile, our current views on the mechanisms of tumor cell dissemination at the beginning of metastasis are still very poor and limited mostly to the *in vitro* studies, due to the challenging nature to observe this cellular process in the mammalian system. The culture studies have proposed two modes of the individual cell invasiveness, the extensive-blebbing-based amoeboid mode and the adhesion-based mesenchymal mode. Ectopic expression of a constitutively active form of *yorkie* (*yki*<sup>35/A</sup>) in the intestinal stem cells of adult *Drosophila* induces the formation of hyperplastic midgut tumors. As the tumor advances, the Yki<sup>35/A</sup>-transformed cells begin to basally disseminate through the basement membrane and the visceral muscle layer towards the body cavity. Each disseminating Yki<sup>35/A</sup> cell forms a polarized, actin-rich leading edge that protrudes through the muscle gap, creating a path for the lagging cell body to extrude out of the intestinal epithelial layer. These Yki<sup>35/A</sup> cells also upregulate the focal adhesion adaptor protein, Talin, at the membrane, and redistribute the localization of Integrin and Talin throughout dissemination, suggesting the significance of focal adhesions assembly in this event. These characteristics observed during *Drosophila Yki*<sup>35/A</sup> cell dissemination highly illustrate the utilization of mesenchymal migration mode by *in vivo* tumor cells. Accomplishing this on-going characterization will establish an *in vivo* model to investigate the novel molecular regulators and the plasticity of *in vivo* cell dissemination modes.

P1114

**Live single cell imaging studies reveal parallel paths for epithelial to mesenchymal transition**

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Recent advances of single cell techniques catalyzed quantitative studies on the dynamics of cell phenotypic transitions (CPT) emerging as a new field. Two grand technical challenges, however, impede further development of the field. Fixed cell-based approaches can provide snapshots of high-dimensional expression profiles but have fundamental limits on revealing temporal information, and fluorescence-based live cell imaging approaches provide temporal information but are technically
challenging for multiplex long-term imaging. The aim of this project is to develop an integrated experimental/computational platform for reconstructing single cell phenotypic transition dynamics. We first developed a live-cell imaging platform that tracks cellular status change through combining endogenous fluorescent labeling that minimizes perturbation to cell physiology, and/or live cell imaging of high-dimensional cell morphological and texture features (Wang et al. Sci Adv., accepted), facilitated by deep-learning based computational image analyses (Wang et al., Computers in Biology and Medicine 108, 133-141 (2019)). With our platform and an A549 VIM-RFP EMT reporter cell line, recorded live cell trajectories reveal parallel paths of epithelial-to-mesenchymal transition (EMT) missing from snapshot data due to cell-cell dynamic heterogeneity. Recognizing that CPTs are examples of rate processes, we introduced transition path analyses and the concept of reaction coordinate from the well-established rate theories into CPT studies, and applied on this EMT process (Wang and Xing, bioRxiv, 2020.01.27.920371 (2020)). We modified a finite temperature string method to reconstruct the reaction coordinate from the trajectories, and reconstruct a corresponding quasi-potential. The potential reveals that the EMT process under study resembles a barrier-less relaxation process. The study emphasizes the importance of live cell imaging in reconstructing cellular dynamics.

P1115

Migratory Transitions and Oncogenic Transformation in Epithelial Cells are Controlled by the Threshold of the Ras/PI3K/ERK Excitable Network

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The Ras/PI3K/ERK signaling network is involved in controlling cell migration and oncogenic progression. There is a lack of systems-level understanding of how the complicated interactions and feedbacks within this network are intrinsically regulated, which largely explains the poor outcome of clinical practices that target various single components of the signaling network. These considerations prompted us to investigate whether the Ras/PI3K/ERK network in human epithelial cells is excitable, and the role of excitability in migratory transition and oncogenic transformation. We demonstrated that, first, traveling waves of Ras and PI3K activation propagate on the basal surfaces of several human mammary epithelial cell lines and drive protrusion formation. These waves can be guided by global and local growth factors, which correlates well with our computational simulation predictions. Second, the Ras/PI3K/ERK network exhibits key features of biochemical excitability, including annihilation of oppositely directed waves, all-or-none responsiveness, and refractoriness to repeated stimuli. Third, abrupt perturbations of phosphoinositide levels, and the activities of Ras, ERK, AKT, and mTORC2 change the properties of waves and protrusions. The positive and negative feedback loops within the network that underlie excitability are delineated. Fourth, the threshold is lowered in the cell with higher wave activity. These observations establish a systems-level mechanism controlling eukaryotic cell migration: cellular protrusions are controlled by an excitable Ras/PI3K/ERK network and growth factors guide cells by locally altering excitability. More importantly, we discovered that Ras transformation leads to de novo or increased wave activity, which contributes to increased protrusive activity of transformed cells. We prove these transformed cells have lowered threshold by showing their higher frequency of ERK pulses and higher
sensitivity to EGF stimulus. Furthermore, the increased wave activity was strongly correlated with metastatic potential across a series of increasingly aggressive breast cancer cell lines (M1~M4 MCF10A cells). These suggest that the enhanced wave and ERK activities control cancer progression. Taken together, our studies suggest a novel view of oncogenic transformation as a shift to a lower threshold of the Ras/PI3K/ERK excitable network. This change in threshold is manifested by an increase in stochastic noise driven activities such as the number and the range of propagating waves and the frequency of ERK pulses. The lowering of threshold most likely leads to the increased migration, macropinocytosis, and proliferation of cancer cells and it possibly can be used to assess cancer severity as well as a target for intervention.

Mechanisms underlying partial versus stable epithelial to mesenchymal transitions
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The ability of carcinoma cells to exhibit plasticity between epithelial and mesenchymal states may be critical for them to adapt to the multiple bottlenecks encountered during metastasis. Such cells acquire mesenchymal properties through the epithelial-to-mesenchymal transition (EMT). Cells that undergo a partial EMT lose epithelial gene expression but maintain their plasticity and can re-express such genes as required. Alternatively, cells can become fixed in a stable EMT whereby they permanently suppress epithelial gene expression and lose plasticity. Alterations in chromatin structure induced by epigenetic mechanisms such as DNA methylation may determine whether cells display phenotypic plasticity or not. We hypothesize that cells achieve a stable EMT by silencing epithelial genes via DNA methylation, whereas other mechanisms regulate the reversible loss epithelial gene expression during partial EMT. Inhibiting DNA methylation in breast cancer cells with stable EMT properties reversed EMT and promoted expression of the epithelial effector CDH1, suggesting that CDH1 and other epithelial genes are epigenetically repressed in a stable EMT. Surprisingly, inhibiting DNA methylation did not prevent epithelial-like cells from undergoing EMT or losing CDH1 expression, suggesting that acute CDH1 loss occurs through independent gene suppression mechanisms. To gain a more comprehensive understanding of which genes become permanently repressed in stable but not partial EMT cells, we generated partial EMT and stable EMT in human mammary-derived MCF10A epithelial cells using TGFβ. Morphological analysis and expression analysis of ~110 genes confirmed that partial and stable phenotypes were attained after TGFβ withdrawal. Furthermore, we identified a cohort of genes that remain repressed in cells exhibiting stable but not partial EMT properties. Collectively, our data suggest that EMT induction slowly reprograms cells, possibly through DNA methylation, by stably repressing a cohort of epithelial genes. Future studies will seek to identify the methylation status of our epithelial gene cohort during partial vs. stable EMT and identify which EMT inducers promote their stable loss. Ultimately, these studies will lead to a better understanding of the gene regulatory mechanisms that determine stable vs. plastic phenotypic states during cancer progression.
Viruses

P1117

**Inhibition of JC Polyomavirus Infectivity by the Retrograde Transport Inhibitor Retro-2.1**

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JC polyomavirus (JCPyV) is a common human pathogen that results in a chronic asymptomatic infection in healthy adults. Upon conditions of immunosuppression, JCPyV spreads to the central nervous system and can cause the fatal demyelinating disease progressive multifocal leukoencephalopathy (PML), a disease for which there are no vaccines or antiviral therapies. In this study, the effects of Retro-2.1 on JCPyV infectivity was studied. Retro-2 is a small molecule that was shown to inhibit intoxication of cells by toxins such as ricin and Shiga-like toxins, and Retro-2.1 is a chemical analog of Retro-2 that is protective at nanomolar concentrations. These inhibitors act by binding to Sec16A, which prevents retrograde transport of toxins from endosomes to the Golgi apparatus and Endoplasmic Reticulum (ER). Retro-2 has previously been shown to prevent retrograde transport of JCPyV virions to the ER, but the effect of Retro-2.1 on JCPyV infectivity is unknown. Dose-response experiments demonstrate that Retro-2.1 inhibits JCPyV infection of tissue culture cells at low micromolar concentrations that are not toxic to these cells. Retro-2.1 was also tested against two other polyomaviruses, the human BK polyomavirus and simian virus 40, and was also shown to inhibit infection at similar concentrations. Following transport to the ER, most polyomaviruses are believed to engage with the host cell quality control machinery, resulting in exposure of the previously solvent-inaccessible minor capsid protein VP2. Immunostaining for VP2 demonstrate that Retro-2.1 acts in a similar fashion as Retro-2 by inhibiting retrograde transport of BKPyV virions to the ER and preventing exposure of VP2. Thus, improved analogs of Retro-2 can inhibit infection at lower dosages than Retro-2 and provide important insight into the cellular pathways used by polyomaviruses to infect cells. Further optimization of these compounds may also lead to effective treatment options for those suffering from JCPyV infection and PML.

P1118

**Characterizing the surface profile of HIV-1-induced T cell syncytia**

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T lymphocyte-based multinucleated infected cells (syncytia) form upon fusion of HIV-1-infected with uninfected CD4+ T cells. HIV-1-induced T cell syncytia have been detected in human patient samples and can comprise up to 20% of infected cells in humanized mice. Importantly, syncytia formation can be induced by multiple strains of HIV-1, including transmitted/founder viruses, i.e. very early upon infection. Even if they form early, however, they stay small, i.e. they don’t grow beyond the four- or five-nuclei stage. If and how these small HIV-1-induced T cell syncytia contribute to virus spread and pathogenesis remains largely unexplored. To gain insight into how they possibly contribute to HIV-1 spread, we have started to compare their surface protein profile to that of infected mononucleated
cells. It is well established that HIV-1 accessory proteins modulate host surface protein expression upon infection. Downregulation of proteins including the viral receptor CD4, BST-2/Tetherin, and NK-T-and -B cell antigen (NTB-A) ensures efficient virus entry and release and also prevents direct sensing by immune cells. Additionally, fusion inhibitory host proteins that prevent excessive syncytia formation at the HIV-1 virological synapse (VS) are also downregulated from the surface, thus precluding that large amounts of these fusion inhibitors can be incorporated into newly released viral particles. Intriguingly, an initial characterization of HIV-1-induced syncytia indicated that, compared to infected mononucleated cells, these entities have altered surface expression of several host proteins. Indeed, we recently documented that syncytia have partially restored surface expression of the fusion inhibitory proteins EWI-2 and tetraspanin CD81 (PMID: 31757023). We predict that altered levels of distinct host surface proteins in HIV-1-induced T cell syncytia impacts the properties of these infected entities, including their fusogenicity, infectivity of released particles, and susceptibility to immune sensing. Notably, we already know that CD81 and EWI-2 inhibit HIV-1-induced cell-cell fusion in a dose-dependent manner, suggesting that increased levels of these proteins in syncytia may decrease their fusogenicity compared to infected mononucleated cells. We are now developing assays to determine how syncytia restore CD81 and EWI-2 surface levels, whether the surface profile of syncytia changes over time, and whether the altered surface profile of syncytia equips them with unique abilities that allow them to spread virus more efficiently than infected mononucleated cells.

P1119

DNA-PK and IFI16 Coordinate an Intrinsic Immune Response to Herpesvirus Infection

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Cellular immunity is tightly regulated to prevent inflammatory disease and cellular dysfunction. In order to ignite an effective antiviral program, cells must first identify foreign molecular signatures and then activate cellular pathways that suppress viral replication and alert neighboring cells. Several immune sensors have evolved to identify viral infections in the nucleus and cytoplasm. Our group has shown that the interferon inducible protein 16 (IFI16) recognizes foreign DNA at the nuclear periphery where it then oligomerizes to serve as a platform for antiviral protein recruitment that suppresses viral transcription and stimulates antiviral cytokine secretion. Previously, we discovered that infection with herpes simplex virus 1 (HSV-1), a nuclear-replicating DNA virus, induces an association between IFI16 and DNA-PK—a master regulator of the DNA damage response (DDR) and an innate sensor for viral DNA in the cytoplasm. This raises the question whether these different sensors of viral DNA cooperate to combat viral infection. To characterize the dynamic association of IFI16 and DNA-PK during HSV-1 infection and understand the downstream protein interactions that execute the antiviral program we performed thermal proteome profiling mass spectrometry (TPP-MS). TPP-MS monitors protein-protein interactions by relying upon the property that associating proteins co-aggregate, which can be measured by quantifying protein abundances after stepwise heat denaturation. TPP-MS readily identified an association between IFI16 and DNA-PK that formed rapidly following HSV-1 nuclear entry, but then disassembled. Functional characterization of this interaction revealed that DNA-PK and IFI16 colocalize at the nuclear periphery following deposition of the viral genome, demonstrating that DNA-PK may contribute to DNA sensing within the nucleus. Inhibiting DNA-PK kinase activity increased viral titers and decreased IFN-β signaling, establishing a role for DNA-PK in the antiviral response to HSV-1. To
understand how DNA-PK suppresses viral infection we performed phosphoproteome characterization of DNA-PK kinase substrates after both viral infection and DNA damage. We discovered DNA-PK-dependent phosphorylation on IFI16 and a substantial enrichment of phosphorylated RNA splicing and metabolism pathway proteins. Indeed, inhibiting DNA-PK kinase activity significantly increased HSV-1 transcription and protein expression. Together these data place the interaction between IFI16 and DNA-PK at the nexus of three distinct cellular pathways—DDR, innate sensing, and RNA metabolism—which must be coordinated in order to initiate an effective antiviral response to viral infection.

P1120

**Insights of Gompertz kinetics into viral - cell interactions**

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Characterizing kinetics of growth phenomena provides a window into their mechanisms of action. The Gompertz equation has been used to provide meaningful insight into growth of embryos, organs, populations, regenerating structures such as limbs, and viral epidemics. As part of a project to understand how increase in viral genetic material in cells affects the mechanisms of infections, we have applied this mathematical model to the coronavirus. The Gompertz equation is a three-parameter growth model and we applied a form of it which incorporates the parameters of asymptote, growth rate and inflection rate. In this pilot study, it was applied to six countries and seven states in order to analyze its descriptive and predictive efficacy. The data for countries in this study was gathered from the Data Repository by the Center for Systems Science and Engineering at Johns Hopkins University. The data for the states in this study was gathered from the New York Times GitHub. This equation has been used successfully to model other viral epidemics, such as the Zika. Excellent fits of the data were obtained for all regressions, with R-Squared >0.98 in all cases. The predictions for the asymptotes stabilized shortly after the inflection points were reached. The sources of variability in the computed parameters were explored regarding implications for mechanisms of transfer of viral genetic materials into cells and populations.

P1121

**Therapeutic targeting of the endoplasmic reticulum resident chaperone BiP for curtailing Kaposi’s Sarcoma-Associated Herpesvirus infections**

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Viruses strictly depend on the host cell machineries to synthesize the massive amounts of proteins required for virion assembly. This high biosynthetic demand takes a toll on the endoplasmic reticulum (ER) and can lead to ER homeostasis disruption. Deviations from ER homeostasis activate the Unfolded Protein Response (UPR), a collection of stress signaling pathways that restore ER homeostasis. The ER-resident chaperone BiP plays a critical role in maintaining ER homeostasis, as it alleviates unfolded protein accumulation while it modulates the activation of the ER-stress sensors IRE1 and PERK. Being a
master regulator of ER homeostasis, BiP is often hijacked by viruses during infection. This is the case during infection by the Kaposi's Sarcoma-Associated Herpesvirus (KSHV), an oncogenic herpesvirus causing significant disease in immunocompromised patients. We have detected BiP upregulation KSHV-infected cells, despite the massive virus-induced downregulation of host protein synthesis. BiP is transcriptionally induced by the membrane-tethered transcription factor and key UPR sensor ATF6. Our data indicate that BiP's upregulation during KSHV infection is not transcriptional and is independent of the ATF6 function. Moreover, we show BiP is critical for completion of the KSHV lytic cycle, as the treatment of cells with HA15, a potent BiP inhibitor, reduces viral DNA replication, late gene expression and virus production. Notably, the UPR activity is scant in KSHV-infected cells, as evidenced by undetectable IRE1 and PERK phosphorylation and activation. In addition, signaling downstream of these sensors is muted in KSHV-infected cells experiencing high levels of chemically-induced ER stress. Our observations suggest a requirement for BiP and an active modulation of the UPR by KSHV to complete the lytic cycle. These results highlight the potential of targeting BiP for the development of next-generation antivirals.

P1122

Role of ATM and DNA-PKcs in early replicative stages of HIV-1 life cycle
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HIV-1 integration results in genomic DNA gaps that are repaired by cellular DNA repair pathways. Previously, we demonstrated participation of the nonhomologous end-joining repair factors (NHEJ) such as Ku70, Ku80 and DNA-PKcs in the post-integrational gap repair (PIR), which is initiated by direct interaction of HIV-1 integrase with Ku70. This interaction leads to DNA-PKcs binding and subsequent activation. Inhibition of DNA-PKcs by small inhibitor Nu7441 decreases the gap repair efficiency and as a result efficiency of cell transduction by HIV-1 based pseudovirus. DNA-PKcs belongs to phosphatidylinositol 3-kinase-related kinase family (PIKKs), including two other DNA damage response kinases: ATM and ATR. To test their contribution to post-integrational repair, we first analyzed the effect of Ku-55933 (ATM inhibitor), AZ20 (ATR inhibitor) and Nu7441 (DNA-PKcs inhibitor, as a positive control) on the 293T transduction efficiency by VSV-G-pseudotyped HIV-1 like particles. Inhibition of ATM or DNA-PKcs but not ATR kinase activity decreased luciferase production (Luc). Interestingly, inhibitors of ATM and DNA-PKcs didn’t affect Luc production in case of pseudovirus with mutant integrase defective in Ku binding and subsequently in PIR. The qPCR data revealed the effect of ATM or DNA-PKcs inhibition on post-integrational DNA repair, but not on reverse transcription or integration. Kinetics of DNA-PKcs and ATM activation were further studied using time of drug addition assay. We found that activation of these kinases occurs simultaneously with integration of viral cDNA (7-14 hpi). Taking into account the fact that the first repaired forms appear with a significant time lag after integration (16-18 hpi), it can be assumed that DNA-PKcs and ATM just initiate PIR, and further repair processes require additional factors. This work was supported by RSF grant 19-74-10021.
Thermal Profiling Reveals Temporal Dynamics of Protein Complex Formation and Dissociation during Human Cytomegalovirus Infection
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Virus replication within host cells proceeds with exquisite spatial and temporal control. To accomplish this, stereotyped biochemical interfaces between virus and host components, such as those mediated by protein-protein interactions (PPIs), are essential. Across diverse virus families, cellular protein complexes are co-opted for virus entry into host cells, virus-stimulated transcription and translation, organelle remodeling, and assembly and egress of infectious viral particles. Similarly, regulation of PPIs is fundamental to host defense mechanisms. However, how viral infection alters the composition and function of host protein complexes on a global scale remains an outstanding question. Here, we investigate the system-wide dynamics of protein complexes throughout infection with the beta-herpesvirus, human cytomegalovirus (HCMV). For this, we use thermal proteome profiling (TPP) and quantitative mass spectrometry to understand PPI and protein complex regulation throughout the course of HCMV infection. We monitor the temporal formation and dissociation of hundreds of functional protein complexes and the dynamics of host-host, virus-host, and virus-virus PPIs during infection. By integrating this thermal shift assay with functional molecular virology and microscopy, we discover pro-viral roles for cellular protein complexes and translocating proteins. Among these, we identify that the trafficking complexes WASH and CCC interact at late stages of infection when virions are assembled. We also show the HCMV receptor integrin beta 1 dissociates from extracellular matrix proteins and is internalized with CD63, which is necessary for virus production. Moreover, this TPP approach facilitates characterization of essential viral proteins, such as pUL52. This study of temporal protein complex dynamics provides insights into mechanisms of HCMV infection and a resource for future biological and therapeutic studies.

Characterizing SARS-CoV-2 S-protein Mediated Cell-to-Cell Fusion
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Characterizing SARS-CoV-2 S-protein Mediated Cell-to-Cell Fusion
Katie Tieu¹, Kenneth Risner², Aarthi Narayanan², Daniel Conway¹ ¹Department of Biomedical Engineering, Virginia Commonwealth University, ²National Center for Biodefence and Infectious Diseases, George Mason University SARS-CoV-2 Spike protein (S-protein) mediated cell fusion plays an important role in the infection and pathogenesis process. Viral protein mediated cell-to-cell fusion is seen in several human viruses such as herpes simplex virus, human immunodeficiency virus, and in the previous severe acute respiratory coronavirus (SARS). We sought to determine the molecular events that contribute to SARS-CoV-2 S-protein mediated cell fusion. epithelial cells, African Green Monkey Kidney (VERO) and Human Embryonic Kidney (293), were transfected with SARS-CoV-2 s-protein to understand the conditions which promote cell-to-cell fusion. Our data demonstrate that a cell-to-cell fusion event can be achieved by the S-protein without additional viral components. It has been identified that a mutation in the S-protein variant D614G has been associated with increased viral load in people with COVID-19 and is the most prevalent strand spreading. We expanded our studies to include the D614G variant S-protein and
we observed that in our test cell types, the D614G variant causes more cell-to-cell fusion events when compared to the wild-type protein. Furthermore, using an FDA-approved small molecule, Maraviroc, our data demonstrate a reduced occurrence of s-protein mediated cell fusion events. This observation raises the possibility of viral spread in a multicellular environment mediated by cell fusion, which can be addressed by Maraviroc treatment. In summary, these data suggest that cell-to-cell fusion mediated by the S-protein is likely to play an integral role in the pathogenesis of SARS-CoV-2 infection and is a valuable target for therapeutic intervention.

P1125

The ACE2 SARS-CoV2 receptor and TMPRSS2/4 coreceptors localize to motile cilia of the respiratory tract and function during viral infections

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Coronavirus disease 2019 (COVID-19) is an ongoing pandemic infection caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a positive-strand RNA virus. The high transmissibility and fatality rates for the virus have raised concerns worldwide. Patients with comorbidities including hypertension, diabetes, and pulmonary disease are highly represented among hospitalized patients with COVID-19 disease, suggesting risk factors for SARS-CoV-2 susceptibility. The ACE2 angiotensin-converting enzyme, a membrane-bound protease and regulator of the renin-angiotensin system (RAS), is a key viral receptor. ACE2 processes Angiotensin II, a hormone controlling vascular constriction and blood pressure, to Ang(1-7) the ligand for the Mas receptor, which counteracts these effects. ACE2 also regulates epithelial cells. The virus enters patients via the upper respiratory tract, a columnar epithelium, rich in multiciliated cells and dotted with mucus-secreting goblet cells. We investigated the expression and subcellular localization of the SARS-CoV-2 receptor ACE2 within the upper (nasal) and lower (pulmonary) respiratory tracts of healthy human donors. We detected ACE2 protein localized dramatically within the multicilia of airway epithelial cells, and by in situ staining of viral RNA, confirmed this to be an early subcellular site of SARS-CoV-2 viral entry during respiratory transmission. We further found that the TMPRSS2 and TMPRSS4 transmembrane proteases linked to the RAS system, and components of the ciliary adenylate cyclase system are also exquisitely ciliary in these tissues. Using patient samples, we found no evidence that the use of angiotensin-converting enzyme inhibitors (ACEI) or angiotensin II receptor blockers (ARBs) increases ACE2 protein expression. Ongoing studies are addressing the importance of multicilia in the infection of epithelia with SARS-CoV-2, the effect of viral spike protein (RBD) binding to ACE2 on ciliary signaling, ciliary beating and endocytosis, the effect of ciliary inflammatory signals on viral entry, and the requirements for ciliary trafficking of ACE2/TMPRSS2/4. SARS-CoV-2 viral infection highlights critical aspects of the cell biology of multiciliated cells and a striking example of how cilia may recognize viruses and activate cellular signals akin to their role in sensory or neuroendocrine signaling.
Length and flexibility enable the extended intermediate of the SARS-CoV-2 Spike protein to capture host cell membranes
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Entry of the SARS-CoV-2 virus, the causative pathogen of Coronavirus disease 2019 (COVID-19), is accomplished by surface spike (S) glycoproteins that fuse the viral membrane envelope with the host cell membrane. Following binding to the host cell ACE2 receptor, the trimeric S protein is thought to undergo a dramatic transition to a key extended intermediate (EI) driven by a “loaded-spring” mechanism, resulting in insertion of the FP into the target membrane. This EI-mediated harpooning is thought the first step in membrane fusion. However, direct experimental evidence for the EI is lacking and the factors determining its ability to capture target membranes is unknown. A major obstacle has been that computational modeling can presently offer relatively little due to time constraints: studying the action of the EI requires following molecular dynamics over long ms-s timescales, well beyond the scope of all-atom computational approaches. Hence it is vital to coarse grain to computationally study these processes. Here we used coarse-grained (CG) MARTIN and ultra-coarse grained (UCG) computational methods to examine the feasibility of the EI and the mechanisms of EI-mediated membrane capture. We constructed representations of the putative EI and measured its interaction with target membranes including the FP penetration depth, which we find is similar to that reported from all-atom simulations of the FP of influenza HA (Baylon et al., 2015). We construct the free energy FP-membrane landscape and we measured the FP pull out force. We measured the time for the EI to capture a host cell membrane for a range of viral-host membrane separations. An interesting conclusion is that critical to the ability of the EI to capture target membranes is its length and flexibility which endows it with a remarkably large capture volume. These quantitative results describe the first stage of S protein-mediated membrane fusion, and will help to assess antiviral strategies based on targeting of the S protein and the fusion process leading to viral entry.

Wednesday, December 16, 2020, 12:00 pm

Actin Dynamics in Endocytosis

P1127

Stresses within the endocytic actin meshwork controls the turnover of fimbrin
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Clathrin-mediated endocytosis is an essential process in eukaryotic cells, transporting molecules from the plasma membrane into the cytoplasm. During clathrin-mediated endocytosis, approximately 60 proteins are recruited in a highly reproducible temporal sequence. Using fission yeast, our laboratory recently showed that, while GFP-tagged endocytic proteins generally remain at endocytic sites for tens of seconds as a population, individual endocytic proteins stay for less than a few seconds, demonstrating they undergo fast turnover. All the endocytic proteins we measured have a dwell time distribution...
containing an individual peak. However, the dwell time distribution of the actin filament crosslinking protein fimbrin contains two peaks. One of these peaks is similar to other endocytic proteins (e.g. actin). The other peak corresponds to a sub-population of fimbrin molecules with a faster turnover. In this study, we hypothesized that one of the peaks of fimbrin dwell time distribution is driven by the fast assembly and disassembly of actin filaments, and the other peak is dependent on mechanical stress on the endocytic actin meshwork. To test our hypothesis, we released the stress in the actin meshwork by genetically weakening the linkages between the filaments and/or the plasma membrane. First, we introduced mutations that produced split fimbrin molecules that can bind actin filaments but are unable to crosslink them. Second, we deleted End4p, the main protein that links the actin meshwork to the plasma membrane. In both approaches, the second peak in fimbrin dwell time distribution disappeared. Our results demonstrate the importance of stress in the turnover of fimbrin at sites of endocytosis.

P1128

Spatiotemporal regulation of actin assembly at endocytic sites by proteins containing SH3 domains and proline-rich motifs

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Networks of branched actin filaments are one of the main force generators in cells and play crucial roles in controlling the shaping and pinching of intracellular membranes. Understanding the finely controlled spatiotemporal regulation of these actin networks and the proteins that regulate actin assembly is therefore critical for understanding the molecular basis of actin-enhanced vesicle formation, such as during clathrin-mediated endocytosis (CME). In yeast cells, actin filament assembly plays an indispensable role in plasma membrane invagination and vesicle scission during CME. Many endocytic proteins contain Src homology 3 (SH3) domains and proline-rich motifs (PRMs) and interactions between these domains are important for precise special and temporal regulation of actin assembly at clathrin-coated pits (CCPs). In mammalian cells, the functions and regulation of actin assembly during CME have not been fully elucidated. Because CME is highly conserved in eukaryotic cells, we hypothesize that similar SH3-PRM interactions may function during mammalian CME. The predicted mammalian homologues of Sla1, CIN85, contains multivalent SH3 domains and PRMs. To avoid overexpression artifacts and to maintain normal cell physiology, CRISPR-Cas9 genome-edited human induced pluripotent stem cell (iPSC) lines endogenously expressing fluorescent protein fusions of up to three of the following proteins, AP2M1, DNM2, ARPC3, and CIN85 were generated. These cell lines provided us with a unique opportunity to rigorously study CME protein functions, spatiotemporal regulation of actin networks and their regulators at CCPs in live cells, and CME adaptation in response to membrane tension changes and stem cell differentiation. By combining total internal reflection fluorescence microscopy (TIRFM) and stochastic optical reconstruction microscopy (STORM), we quantitatively analyzed the abundance, detailed spatial distribution, and dynamics of these proteins at CCPs. We observed asymmetrical actin nucleation at CCPs shortly before endocytic vesicle scission, and that initiation of actin nucleation is tightly correlated with CIN85 recruitment to CCPs. We further analyzed SH3 or PRM mutants of CIN85. Our data suggest essential but partially redundant roles of these domains in CIN85’s recruitment to CCPs. To determine CIN85’s functions in CME, we studied actin assembly and overall CME dynamics in the wild-type, CIN85 knockdown or CIN85 overexpressing cells, and found CIN85 level is negatively correlated with CME lifetime. Moreover, upon high membrane
tension, we observed increased CIN85 recruitment and actin nucleation at CCPs. These results provide important insights into the regulation of actin networks and roles for CIN85 in actin assembly at CCPs in human cells.

P1129

**WIP/Verprolin Enhances Actin Assembly via WASp/Wsp1 at Endocytic Sites in *S. pombe***

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Branched-actin networks nucleated by the Arp2/3 complex provide the force needed within cells to rearrange the plasma membrane and carry out essential processes including cell motility and endocytosis. The Arp2/3 complex is activated by Nucleation Promoting Factors (NPFs), such as Wiskott-Aldrich Syndrome protein WASp. Human patients with WASp mutations develop Wiskott-Aldrich Syndrome WAS, a severe immune disorder. Mammalian WASp is degraded in the absence of the WASp Interacting Protein (WIP), resulting in a WASp mutant-like phenotype, making it difficult to determine additional roles for WIP outside of protecting WASp from degradation. However, in the fission yeast *S. pombe*, WASp homolog Wsp1 remains stable in the absence of WIP homolog Vrp1. This system provides us a unique environment to understand how WIP contributes to actin assembly. In this study, we investigate mechanisms of branched-actin assembly at endocytic actin patches in *S. pombe*. Fission yeast has four known NPFs including Wsp1 and the class-1 myosin Myo1. Wsp1 recruits Vrp1 to actin patches, where both proteins interact with Myo1 forming a transient complex. Vrp1 has three characterized domains including two actin binding WH2 domains, a central proline rich domain (PRD) which can bind Myo1, and a C-terminal Wsp1 binding domain (WBD). Vrp1 is capable of enhancing Myo1 NPF activity *in vitro*; however, Vrp1’s physiological role is still unknown. To uncover the role of Vrp1 on actin assembly *in vivo*, we created a series of Vrp1 mutants lacking individual domains. These mutant genes were expressed under the endogenous *Pvrp1* promoter introduced into cells expressing endocytic proteins tagged with monomeric GFP. Endocytic protein dynamics were examined and analyzed using quantitative spinning disk confocal microscopy. We discovered that Vrp1 plays an important, novel role in actin assembly. In the absence of Vrp1, accumulation of actin in endocytic patches and the distance travelled by endocytic structures are significantly reduced. Surprisingly, these observations were independent of Vrp1’s Myo1-binding PRD or the actin binding WH2 domains. Further, while loss of Myo1 NPF activity had no impact on patch actin assembly or vesicle internalization, removal of Wsp1 NPF reduced patch actin assembly and distance traveled by endocytic vesicles to the same extent as cells lacking Vrp1. This suggests Vrp1 enhances actin assembly via Wsp1, providing the force needed to properly internalize endocytic vesicles. Further, we developed an assay to measure actin assembly *ex situ* and observed the Vrp1 WBD, but not the PRD or WH2 domains, was necessary to activate Wsp1-mediated actin assembly *in vivo*. This data provides novel evidence that WIP homolog Vrp1 regulates branched-actin assembly by activating Wsp1 NPF activity.

P1130

**A Promising New Cell Penetrating Peptoid Induces Actin Reorganization and Macropinocytosis**

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Cell-penetrating peptides (CPPs) are emerging drug delivery vehicles because of their capacity to induce efficient cellular uptake of molecules that otherwise do not enter cells. Amongst cationic CPPs, the guanidinium-rich transporters (GRTs) emerged as efficient delivery vehicle given their increased plasma stability. Our group recently developed diversified GRTs bearing conformationally restricted guanidinium display scaffold exploiting the chemical space of glycosaminoglycans (GAGs) at the plasma membrane with the goal to study the impact on cellular delivery and selectivity. We identified PGua₄, a 5 amino acid GRT composed of 8 guanidines, that showed low cytotoxicity, high cellular penetration dependent on cell surface GAG and cellular selectivity, compared to the nona-arginine (R9), a well characterized CPP. In this study, we have investigated the cellular uptake mechanisms of PGua₄ coupled to fluorescein, by live cell confocal microscopy using various internalization inhibitors and selective Rab protein markers of intracellular compartments. PGua₄ is partly internalized through clathrin-dependent and macropinocytosis pathways and is also found in early (Rab5), late (Rab7) and recycling (Rab11) endosomes as well as in the cytoplasm and nucleus. PGua₄, unlike R9, escapes from the endosomes into the cytoplasm which would allow the cargoes to reach their therapeutic targets. Interestingly, we also observed that PGua₄ induced a remodeling of the actin network at the cell periphery. Concomitantly, using a GST-pull down assay, we showed that PGua₄ induces a rapid and prolonged activation of Rac1, a key GTPase involved in actin reorganisation and macropinocytosis. In conclusion, our data indicates that PGua₄ is a novel CPP internalized by multiple pathways that quickly activates the intracellular signaling pathway leading to actin organization for macropinocytosis and thus, could facilitate the intracellular delivery of therapeutic compounds.

P1131

**CYRI-A is recruited to macropinocytic cups and mediates integrin uptake, limiting invasive migration.**
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Cell migration underlies many fundamental biological processes from development to cancer progression, yet our understanding remains incomplete. A recent discovery from our lab have characterised a novel negative regulator protein family of the Scar/WAVE complex which we termed CYRI. The protein family acts by binding and sequestering active RAC1 to control membrane dynamic and actin cytoskeletal organisation. Even though biochemical analysis has shed some light on the mode of action of CYRIs, their cellular localisation and membrane interaction kinetics remain unknown. In mammals, the CYRI family contains two evolutionarily conserved paralogs CYRI-A and CYRI-B, of which the former is completely unknown. Using the combination of biochemical analysis and genetic manipulation with siRNA and CRISPR-Cas9, we discovered that CYRI-A is able to directly bind active RAC1 in a similar fashion as CYRI-B, but at a 10 times higher affinity. In cellulo, CYRI-A is able to rescue the effects of CYRI-B deletion and both CYRI cooperatively regulate cell shape and migration. Using an internal GFP tagging strategy along with super-resolution microscopy, we discover that CYRI-A is dynamically localising to large endocytic vesicles, which are confirmed to be macropinosomes through the use of dextran uptake assay. By studying the spatiotemporal dynamic of CYRI-A at the macropinocytic cups, we place CYRI-A downstream of PIP3, RAC1 and actin but upstream of the early endosomal marker RAB5A. We believe CYRI-A acts as a local inhibitor of the RAC1-Scar/WAVE-actin signalling axis at the endocytic cup to aid for the completion of macropinosomes. Furthermore, we show...
that CYRI-A and CYRI-B contribute to the regulation of integrin homeostasis in cancer cells. CYRI-positive vesicles contain α5β1 integrins and disruption of CYRI signalling leads to an increase in surface level of α5β1 integrins in cancer cells due to reduced internalisation. Increased level of integrins lead to increased cell migration on 2D substrate, invasive capacity and growth in 3D matrix. Inhibition of integrins or their downstream effector FAK suppresses these phenotypes. Overall, we identified CYRI-A as a novel local actin modulator specifically at the macropinocytic cups and the involvement of the CYRI protein family in the regulation of integrin trafficking and cancer cell migration and invasion.

P1132

Mechanisms of actin force production in clathrin-mediated endocytosis revealed by integrating mathematical modeling with in situ cryo-electron tomography

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Many essential cellular processes, including clathrin-mediated endocytosis (CME), depend on the function of actin filament networks. During CME, the plasma membrane is deformed, forming clathrin-coated vesicles (CCVs) containing cargo. Membrane remodeling is supported by actin filament assembly, but its mode of function remains elusive. We previously used an experimentally constrained mathematical model to find that a minimal endocytic actin network can self-organize, bend, and produce sufficient force at sites of CME for pit internalization (Akamatsu et al., eLife 2020). The study revealed gaps in knowledge of the structural organization of actin filaments in CME necessary to understand its endocytic function. Here we used cryo-electron tomography (cryo-ET) of intact mammalian cells to directly visualize networks of individual actin filaments at CME sites and CCVs, and used mathematical modeling to identify their mechanistic functions. Surprisingly, actin networks at CME sites consisted of both branched and unbranched filaments. Endocytic actin filament lengths were exponentially distributed (90 ± 80 nm), which was predicted by simulations of our previously published model and can be explained by stochastic filament capping. In contrast to previous studies, actin filaments localized not only in short branches around the neck of the pit but also in long filaments stretching between the coat and the plasma membrane. The branched filaments were arranged in multiple discrete clusters, in agreement with model simulations, implying that actin networks arise from several distinct “founding” mother filaments. CME actin branched at a 70 ± 8° angle, indicating stiffer branch junctions than previously assumed. Simulations suggested that endocytic success is robust to these variations. We determined actin filament polarity. Branched filament plus ends were oriented to allow for plasma membrane deformation and CCV transport. Finally, we identified long proteins ~60 nm in length resembling the actin-CME linker Hip1R, both in the clathrin-coated area and in the neck of the pit. This neck localization of Hip1R linkers might direct actin polymerization-mediated force generation toward the neck of CME sites for vesicle scission. We are currently testing this hypothesis using mathematical modeling. Taken together, our results reveal the complex actin filament organization at endocytic sites in unprecedented structural and mechanistic detail. By combining mathematical modeling and in situ cryo-ET, deeper insights into actin mechanism during CME were achieved than either approach alone. Such a collaborative approach is necessary going forward to gain a mechanistic understanding of the interplay of ensembles of force-producing protein complexes in cellular processes.
Paclitaxel-induced micronucleation activates pro-inflammatory cGAS-STING signaling in triple negative breast cancer

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Paclitaxel (Taxol™) is a blockbuster chemotherapy used for solid tumors including breast cancer. Nab-paclitaxel plus PD-L1 inhibitor atezolizumab is now approved for select triple-negative breast cancers (TNBC) based on increased survival compared to nab-paclitaxel alone in TNBC. We hypothesized that there may be an interaction by which taxane therapy is immunogenic. Paclitaxel induces aberrant cell division on multipolar spindles, which frequently leads to formation of nuclear fragments resembling micronuclei. Here, we test the hypothesis that taxane-induced micronuclei activate cGAS-STING in triple negative breast cancers. We evaluated the impact of clinically relevant doses of paclitaxel on TNBC cell lines including MDA-MB-231 to generate micronuclei and to activate the cGAS-STING and interferon-beta pathways. Furthermore, we co-cultured MDA-MB-231 and THP-1 cells to measure the impact of paclitaxel on polarization of THP-1 macrophages. We employed CRISPR-mediated cGAS knockout to assess its role in mediating these effects. Finally, we tested the effect of neoadjuvant paclitaxel (80mg/m² qw, 16 treatments/4 cycles) in 4 patients for formation of micronuclei and tumor infiltrating lymphocytes (TILs) 20 hours after the third dose of paclitaxel by H&E. We observed that post-mitotic cells often contain micronuclei. cGAS recruitment is detected in some micronuclei, more commonly in multinucleated cells. With three days paclitaxel, the cGAS-positive cell population increases from 9% +/- 1.3 to 34% +/- 2.4 in MDA-MB-231. We observed similar trends in other TNBC cell lines with spindle poisons exhibiting the greatest effects compared to other chemotherapies and radiation. Synthesis of 2’3’-cGAMP and interferon-beta is increased after paclitaxel, comparable to levels achieved using ionizing radiation. Upregulation of interferon stimulated genes and phospho-proteins also supports STING activation, which was abolished upon cGAS knockout. Conditioned media and co-culture of MDA-MB-231 in paclitaxel polarize THP-1 macrophages from M0 to M1 phenotype. Furthermore, we observed an increase in micronuclei and TILs after paclitaxel for 3 of 4 patient samples. We conclude that physiologic levels of paclitaxel can generate micronuclei that activate the cGAS-STING pathway in breast cancer cell lines and induce a pro-inflammatory interferon-beta driven response, which may present a post-mitotic mechanism for paclitaxel-mediated cancer cell death. These data may provide the mechanistic underpinning for the high efficacy seen in combining taxanes with immunotherapies.
P1134

**Chromosomal instability sensitizes tumors to multipolar divisions induced by paclitaxel**

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Paclitaxel (Taxol™) is a cornerstone of cancer treatment. However, its mechanism of cytotoxicity is incompletely understood and not all patients benefit. We conducted an IRB-approved biomarker study in which a research biopsy was procured 20 hours after the first weekly dose of 80 mg/m² paclitaxel in newly diagnosed HER2-negative breast cancer for which neoadjuvant chemotherapy was indicated. We find that paclitaxel concentrations are substantially higher in primary breast cancers than in serum and that intratumoral concentration does not correlate with response, arguing against drug efflux pumps as a major mechanism of resistance. Despite relative paclitaxel accumulation, primary breast cancers do not acquire sufficient intratumoral paclitaxel to induce mitotic arrest. Instead, clinically relevant concentrations induce multipolar mitotic spindle formation. However, the extent of early mitotic multipolarity does not predict patient response. Multipolar spindles focus into bipolar spindles prior to division at variable frequency, and maintaining multipolarity throughout mitosis is critical to induce the high rates of chromosomal instability (CIN) necessary for paclitaxel to elicit cell death. Moreover, pre-existing CIN potentiates cytotoxicity from paclitaxel-induced multipolar spindles in cell culture and correlates with response in taxane-treated metastatic breast cancer patients. These results support the use of baseline levels of CIN as a predictive biomarker for paclitaxel response and suggest that agents that increase CIN or maintain multipolar spindles throughout mitosis will improve the clinical utility of paclitaxel.

P1135

**Cytotoxic assessments and RNAseq interpretation of Cyanoximate-treated cancer cells**

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Chemotherapy remains one of the most effective treatment plans for several cancer types. The idea of searching for chemical compounds with better efficacy and minimal side effects prompted us to investigate the effects of a group of newly synthesized metal based chemical compounds called cyanoximates on HeLa cervical and ML-1 thyroid human cancer cells. We established that metal cyanoximates reduced cell viability via apoptosis and specifically, Pt(DECO)₂ was most effective among these new cyanoximates. Our results also provide evidence that Pt(DECO)₂ can be an alternative agent for Cisplatin-based chemotherapy by using transcriptomic analysis to highlight the mechanism of action of Pt(DECO)₂ against cancer cells.
PTER ABSTRACTS

P1136

Identifying Cellular Pathways in Response to Anticancer Drugs, Cisplatin and Carboplatin, using Genomic Screening Analysis

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Platinum-based anticancer drugs are effective chemotherapeutic agents, most commonly used in the treatment of ovarian, prostate and breast-related cancers. Cisplatin, as the prototype of platinum drug, and Carboplatin, a second-generation platinum drug, are both routinely used for cancer treatment; yet little is understood regarding the pathways involved in the cellular response to these drugs. The work done in our lab explores the cellular sensitivity and resistance elicited by these two drugs. To identify the genes involved in cellular response to Cisplatin and Carboplatin, we performed growth-fitness based screening assays using fission yeast, Schizosaccharomyces pombe, as the model organism for this screening because of its high degree of conservation with complex eukaryotes, as well as its well-annotated genome. Specifically, an S. pombe haploid deletion library consisting of 2851 deletion strains was assessed for growth of each deletion mutant, in the presence and absence of the individual drugs. The resulting colony growth of each strain was then digitized into quantifiable values using the CellProfiler software. The normalized growth score values (GSVs) of mutant strains were determined by comparing the growth of each mutant strain with those of the wild-type strains, under the same growth conditions. These analyses allowed us to identify 105 and 214 strains as sensitive and 131 and 97 deletion strains as resistant to Carboplatin and Cisplatin, respectively. To determine the biological pathways involved in the cellular response to these drugs, the gene sets from sensitive and resistant strains were further analyzed using the Princeton Gene Ontology (GO) finder. Interestingly, increased cellular sensitivity against both Carboplatin and Cisplatin was observed in strains with deletions in genes involved in protein sorting, membrane transport, cytokinesis as well as chromatin remodeling and silencing. Intriguingly, chromatin remodeling was also identified as a common biological process when analyzing the strains resistant to both drugs. Remodeling chromatin structure is one of the known pathways that cells use to develop chemotherapy resistance. Our results support the involvement of this pathway in platinum-based drug resistance and toxicity. Further research on these specific pathways bears potential to help improve cancer therapy through reducing cellular toxicity and resistance to these platinum-based treatments.

P1137

Genomic Analysis of Cellular Response Pathways to the Platinum-based Anticancer Drugs, Oxaliplatin and Dicycloplatin

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Platinum-based anticancer drugs are known to induce cancer cell death via DNA damage. Oxaliplatin, one such platinum drug, was clinically approved over two decades ago, as an effective treatment for...
colorectal cancer. On the other hand, Dicycloplatin (DCP), a new supramolecular platinum-based antitumor drug, has been in clinical use only since 2012 for the treatment of non-small-lung carcinoma and prostate cancer. Despite their routine clinical application, the mechanism of action of these drugs as well as the resulting sensitivity or resistance to these drugs is not yet well understood. We performed a genome-wide growth-fitness based assay against Oxaliplatin and Dicycloplatin, to identify the genes involved in the platinum-based drug response. We used the fission yeast, *Schizosaccharomyces pombe*, as the model organism for this genomic screen because of its conservation with complex eukaryotic systems including humans and its well-annotated genome. The *S. pombe* haploid deletion library consisting of 2815 strains, was screened for growth on the drug positive plates as well as the control plates. The colony growth for all strains was digitized using the CellProfiler software. After normalizing the growth of each strain using growth score value (GSV), we identified 62 and 579 deletion mutants as sensitive, as well as 12 and 163 deletion strains as resistant to Oxaliplatin and Dicycloplatin, respectively. A gene ontology analysis was conducted on the gene sets from both sensitive and resistant strain categories to identify significantly associated biological processes. The results of these screenings taken together, provided us some interesting insights; notably, deleting genes involved in processes such as DNA repair, DNA metabolism, carbohydrate metabolism, membrane transport, and chromatin remodeling, increased the cellular sensitivity against both drugs. Genes deleted in resistant strains from both drug screenings were identified to be enriched in processes involved with protein metabolism. Our data from this genomic analysis provides a deeper understanding of the biological processes implicated in the cellular response to platinum-based drugs, and reveals potential molecular targets for improving and enhancing their chemotherapeutic effects.

P1138

Uncovering the mechanistic basis of MAGEA5/A10 driven resistance to chemotherapeutic drugs

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Melanoma Antigen Genes (MAGEs) are defined as cancer-testis antigens due to their unique expression pattern. There are two families of MAGEs, Type I and Type II. Type I MAGEs are expressed in the testis and in no other normal somatic tissue and then re-expressed in many cancers. Type II MAGEs on the other hand, are ubiquitous in their expression. The MAGE-A family are members of the type 1 family located on the X chromosome. Type I MAGEs, considered to be true cancer-testis antigens are all located on the X chromosome. Previous studies show that reversible DNA methylation of CpG sites upstream of MAGE-A promoters regulates their expression. Many members of the MAGE1 subfamily cause increase in proliferative phenotypes. However, MAGEA5 and MAGEA10 expressing cells, demonstrated resistance to high doses of chemotherapeutic agent 5-flourouracil. We aim to understand the mechanism behind this resistance. We will perform RNA-Sequencing on cells expressing MAGEA5/A10 and control cells and determine protein binding partners of MAGEA5/A10 using IP-Mass Spectrometry. In addition, we will test the hypothesis that in the heterogenous tumor cell population, MAGEA5/A10 is expressed in the cancer "stem cells" and that is responsible for driving chemoresistance. We will also test resistance of these cells to other chemotherapeutic agents such as taxols.

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Cell-Cell Interactions and Tissue Development

P1139

**Micropatterning of EM Grids for Cryo-electron Tomography of Endothelial Cell-cell Junctions**

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Endothelial cell-cell contacts contain adherens junctions that are important for controlling vascular permeability. While it is known that altered junctional organization can lead to vascular leakage, the detailed macromolecular organization at endothelial cell-cell junctions is poorly understood. Cryogenic electron tomography (cryo-ET) is increasingly being used to investigate macromolecular complex structure and organization inside cells. At present, cryo-ET data acquisition suffers from low throughput, due principally to the improbability of a cell fortuitously positioning the cellular structure of interest over a region of the support that is suitable for imaging. Extracellular matrix (ECM) micropatterning of electron microscopy (EM) grids via photo-micropatterning has emerged as a method to position and confine cells to specific shapes on EM grids for *in situ* cryo-ET (Engel et. al., JMM 2019). We have extended this technique in order to generate a method for optimally positioning endothelial cells on EM grids so as to enable high-throughput imaging of cell-cell junctions. Specifically, our approach increases the number of optimally positioned cell-cell junctions per grid, and increases distance between the junctions and the thicker cell nuclei to promote the assembly of junctions that are sufficiently thin for imaging. The resulting sample preparation pipeline enabled the observation of a number of interesting phenomena at endothelial cell-cell contacts by cryo-ET, demonstrating the suitability of this technique for *in situ* structural studies of cell-cell adhesive contacts.

P1140

**Calcium signaling contributes to epithelial tight junction remodeling during cell shape changes**

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Cell shape changes challenge epithelial cell-cell adhesion and barrier function. In order for an epithelium to serve as an effective barrier, tight junctions must be remodeled during events that cause cell shape changes, such as morphogenetic movements and cytokinesis. Using live imaging in the epithelium of gastrula-stage *Xenopus laevis* embryos, we have shown that junction elongation correlates with local loss of tight junction proteins, resulting in a leaky barrier. These leaks are rapidly repaired by localized, transient activation of RhoA – termed “Rho flares” (Stephenson et al., 2019) – which promote actomyosin-mediated reinforcement of tight junction proteins. However, the mechanism underlying local activation of Rho flares is unknown. Here, we show that a transient increase in intracellular calcium precedes activation of Rho flares and is localized to the site of Rho flares. Further, using drugs to deplete calcium inside the cell, we show that calcium is required for efficient repair of tight junction breaks in a Rho dependent manner. Because Rho flares are associated with junction elongation and a local apical plasma membrane deformation, we propose that mechanically-triggered calcium channels may be an
important mechanism by which cells sense and respond to transient leaks in barrier function. Piezo1 is a mechanosensitive calcium channel (MSC) that senses the membrane tension caused by stretch and curvature of the membrane and allows calcium ions to pass through the membrane. We found that Piezo1 localizes to apical cell-cell junctions, and upon inhibiting Piezo1 (using the antagonist GsMTx4), the frequency of barrier breaches and the frequency of Rho flares increases significantly compared to controls. Further, we show that mechanosensitive calcium channel mediated calcium influx is required for efficient reinforcement of ZO-1 and sustained activation of Rho flares, suggesting that the increase in the frequency of barrier breaches is due to impaired repair of ZO-1 loss. We are currently testing the effect of Piezo1 knockdown on the dynamics of calcium influx during Rho flares and the effect on barrier function. Overall, this work will shed light on the basic molecular mechanisms that convert a mechanical cue into a local biochemical signal to regulate barrier function in the vertebrate epithelium.

Reference:

P1141

**The septate junction protein Bark beetle is required for *Drosophila* intestinal barrier function and homeostasis**

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In the intestine, the epithelial barrier is maintained by tight junctions (TJs) in mammals and septate junctions (SJs) in insects. The intestinal barrier allows para-cellular flow of water, ions and nutrients across the epithelium, while maintaining food matter and microbes inside the intestinal lumen. Age-related loss of intestinal barrier function has been found across multiple species, including *Drosophila melanogaster* and humans, yet the age-related causes of barrier dysfunction remain unknown. The tricellular junction (TCJ) is a specialized region of the SJ where three adjacent cells meet. Previous studies indicated that the TCJ protein Gliotactin (Gli) accumulates in the cytoplasm, rather than at the TCJ, in intestines from old flies, and depletion of Gli leads to increased intestinal stem cell (ISC) proliferation in young flies, similar to what occurs with age. In the embryo, the TCJ protein Bark beetle (Bark) is required to recruit Gli to the TCJ; therefore, we hypothesized that Bark would be required for maintenance of intestinal homeostasis and barrier function, similar to Gli. Indeed, depletion of Bark from differentiated, absorptive enterocytes in a young fly led to loss of the intestinal barrier and an increase in ISC proliferation. In addition, our data show that Bark, similar to Gli, becomes mis-localized with age. However, the mis-localization of Bark and Gli differ, with Bark accumulating along the bicellular junction, while Gli accumulates in the cytoplasm. Future studies will focus on understanding the mechanisms leading to Bark and Gli localization and turnover in the *Drosophila* intestine. Our work on the mechanisms leading to loss of the intestinal barrier will enhance treatment for age-related gastrointestinal disease, such as cancer.
Using Electron Microscopy to Examine Bone Formation and Density in Zebrafish

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It is possible to study the mechanisms underlying the development of bone in zebrafish because they can regenerate missing caudal fin tissue. This process of bone growth is affected in several fin length mutants, including the short fin (sof) and long fin (lof) mutant. As bones grow, new segments are added to the distal tip of the fin. In the sof mutant, the number of segments produced is similar to wild type, however, the size of the segments is shorter; whereas, the lof mutant produces segments similar in size to wild type, but the number of segments is greater. The sof mutants develop short fins due to mutations in the gap junction gene connexin43 (cx43). Gap junctions serve as intracellular passageways for the exchange of molecules between cells, including the exchange of signals between osteoblasts. Gap junctional intercellular communication (GJIC) between these cells may be responsible for regulating the length of the bony segments. Previously, we have shown that the level of cx43 mRNA is reduced in short fin mutants and the fins that develop are half the length of wild type fins. In addition, the level of cell proliferation is reduced in the regenerating cells of the short fin mutant. Targeted gene knockdown of cx43 in the regenerating fin restored the segment length and cell proliferation defect in the sof mutant. The level of cell proliferation in the regenerating fin may be regulated by the amount of communication via gap junction channels. This in turn would regulate the growth of new bone. Therefore, if bone growth is regulated by channel communication, then defects in Cx43 may affect the structure of the channel and GJIC. Bony fin rays in regenerating tissue were observed using electron microscopy (EM), Energy-dispersive X-ray spectroscopy (EDS) and Quantitative backscatter imaging (qBEI). In order to visualize bone more directly in the regenerating fin, several methods of removing the skin tissue were employed. Electron microscopy allows us to observe any chemical composition differences, including calcium and bone density, that may exist between newly formed bone and old bone. As humans enter microgravity environments such as during space exploration or as humans age, overall bone density decreases. Bone composition studies in newly formed bone tissue can help us better understand the self-repairing nature of fracture healing and may help us to find ways to prevent or reduce bone density in humans on earth as well as in space.

Multiple stages of germ cell differentiation in Drosophila testes require gap junction-mediated soma-germline communication

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Gametogenesis is a developmentally conserved process in animals that requires coordinated signaling between germ cells and somatic cells. It was shown in Drosophila melanogaster that communication between soma and germline regulates cell proliferation and differentiation, failure of which can lead to infertility or cancer. Gap junctions (GJs) mediate cell-cell communication, as they allow the passage of small molecules between cells. GJs in Drosophila are made of innexin proteins that are functionally homologous to vertebrate connexins. We previously showed that Innexin4/Zero population growth (Zpg) mediates bi-directional signaling between soma and germline. Flies lacking zpg expression are
sterile due to the arrest of germ cell differentiation at an early stage and impaired germ cell maintenance. Our hypothesis is that specific signals pass through GJs, consisting of Zpg on the germline side and Inx2 in the soma, to control the developmental program of gametogenesis. To explore this hypothesis we used insight into the protein structure of innexins to design mutations aimed at selectively perturbing the passage of different cargos to different extents. Our goal is to identify mutations that block some cargos but not others. Using a genetic structure-function analysis approach, we replaced the endogenous Zpg protein in the testes with mutated versions of the protein and analysed germline and soma development by performing immunofluorescence stainings and fertility assays. To date we made and analyzed nearly two-dozen different Zpg mutants. While many led to arrest of early germ cell development, we identified a subset that disrupt specific stages of germline or soma development. E.g. flies with mutations in residues in the channel pore which, according to 3D protein homology modeling data, are predicted to directly interact with cargo or to regulate pore permeability exhibited normal early germline development, but entry to meiosis was strongly inhibited, resulting in reduced fertility or sterility. To further analyze the function of GJ-mediated soma-germline communication, we are using fluorescent biosensors to find out which cargos pass through the junction formed by Zpg and Inx2. Preliminary data suggests a misregulation of intracellular pH in somatic cells of zpg mutants. Our work shows that specific signals that pass through GJs regulate the transition between different stages of gametogenesis.

P1144

Coordinated waves of cell separation during wound healing of larval zebrafish epidermis

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The ability of epithelial cells to coordinate at the tissue level is central to physiologically important activities such as wound healing and regeneration. Wound healing in particular consists of a series of collective processes, including reepithelialization, in which epithelial cells migrate to cover damage and protect the underlying tissue. The mechanical and chemical signals that initiate and maintain migration during reepithelialization have been well studied, but less is known about the collective behaviors associated with the end of reepithelialization, when cells must stop moving and re-form an epithelium. Here we report a collective process at the end of reepithelialization in wounded epidermis in zebrafish larvae. The larval epidermis is bilayered, with a basal cell layer resting on a collagenous extracellular matrix and a superficial layer that seals the embryo with tight junctions. Basal epidermal cells are known to actively migrate towards injury. We have discovered that as basal cells stop migrating, they pull apart from one another (increasing apparent inter-cell distance), while the superficial cell layer remains intact. This behavior contrasts with the expectation that cells no longer migrating might adhere together more tightly as they reform an intact epithelium. Cell separation propagates through the tissue in a wave, starting at the wound site and extending several hundred microns into the tissue. The onset of separation of a cell from its neighbors coincides with an increase in the number of intracellular vesicles, possibly endocytic or secretory in nature. Cells remain separated from each other for hours after the wound healing process has completed, though they do eventually reconnect. Observations of cell junctions with confocal and electron microscopy suggest that cells remain connected via thin tethers containing desmosomes and adherens junctions, while the bulk of cell cytoplasm remains >1µm apart.
from neighboring cells. Intriguingly, this process appears to be partially dependent on myosin II activity, as cells treated with blebbistatin do not separate as dramatically. Work is ongoing to understand the physical driving force—whether accumulation or release of tension at cell-cell junctions—that underlies this separation behavior and might explain the coordinated wave-like propagation of separation through the tissue.

P1145

**Arp2/3 maintains cell-cell junctions to preserve epithelial tissue integrity in gut homeostasis**

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The gut epithelium is a selective tissue barrier with the largest surface area in the body, and is also one of the fastest-renewing tissues in adult mammals (3-5 days). How gut epithelial integrity is maintained amid this dynamic turnover remains largely unknown, despite its physiological and clinical importance. Here, using pharmacological, genetic and force manipulation experiments in mice, we discovered that strong, tissue-intrinsic tensile forces mechanically challenge the integrity of epithelial cell-cell junctions along the intestinal villi. We found that tissue integrity is critically dependent on the actin-related protein 2/3 (Arp2/3) complex, which is localized at the apical cell-cell junctions. Targeted depletion of Arp2/3 in the gut epithelium of adult mice resulted in tissue fracturing along the apical cell adhesions, a pathological increase in intestinal permeability, and an inflammatory response. The markers of tight and adherens junctions and perijunctional F-actin belts were significantly decreased in the Arp2/3-depleted tissue, whereas cell-matrix adhesions were not compromised. Also, Arp2/3-depleted tissue rapidly fractured upon junctional laser ablations and mechanical stretching, indicating defective transmission of tensile forces. We propose that Arp2/3 has a critical role in maintaining cell-cell junctions to protect the intestinal epithelial barrier from mechanical stresses.

Cellular Microtubule Organization

P1146

**Chlamydia Hijacks ARF GTPases To Coordinate Microtubule Posttranslational Modifications and Golgi Complex Positioning**

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The intracellular bacterium Chlamydia trachomatis develops in a parasitic compartment called the inclusion. Posttranslationally modified microtubules encase the inclusion, controlling the positioning of Golgi complex fragments around the inclusion. The molecular mechanisms by which Chlamydia coopts the host cytoskeleton and the Golgi complex to sustain its infectious compartment are unknown. Here, using a genetically modified Chlamydia strain, we discovered that both posttranslationally modified microtubules and Golgi complex positioning around the inclusion are controlled by the chlamydial inclusion protein CT813/CTL0184/InaC and host ARF GTPases. CT813 recruits ARF1 and ARF4 to the inclusion membrane, where they induce posttranslationally modified microtubules. Similarly, both ARF isoforms are required for the repositioning of Golgi complex fragments around the inclusion. We demonstrate that CT813 directly recruits ARF GTPases on the inclusion membrane and plays a pivotal role.
role in their activation. Together, these results reveal that Chlamydia uses CT813 to hijack ARF GTPases to couple posttranslationally modified microtubules and Golgi complex repositioning at the inclusion.

P1147

**Microtubule polarity sorting in the axon: motors and crosslinkers**

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We previously proposed that the nearly uniform plus-end-distal polarity orientation of microtubules in the axon could be established and preserved by a simple polarity-sorting mechanism in which microtubules are transported in the axon, plus-end-leading, by cytoplasmic dynein. In this manner, the axon would be populated by plus-end-distal microtubules while any minus-end-distal microtubules that might arise would be cleared back to the cell body. Experimental evidence from our studies was consistent with such a mechanism but suggested that additional elements are needed to fully explain the data. In particular, most of the microtubule mass in the axon appeared to be stationary at any given moment with the observable movements being restricted to microtubules shorter than ten microns in length. Transport of these short microtubules was jerky, with occasional backward steps. Computational modeling demonstrated that the data could be explained by the addition of static crosslinker proteins that impede the movement of microtubules in a length-dependent manner and by the participation of a plus-end-directed kinesin capable of transporting microtubules oppositely to cytoplasmic dynein. Forces generated by this kinesin, which we suggested might be kinesin-1, must be overcome to ensure appropriate polarity sorting. Insufficient crosslinking and/or too much activity of the kinesin, according to the model, would obstruct normal polarity sorting by cytoplasmic dynein and create abnormal microtubule movements that might lead to microtubule polarity flaws. Consistent with the predictions of the model, we report here (in primary cultures of rat hippocampal and sympathetic neurons) that experimental depletion of TRIM46 or PRC1, two different microtubule-crosslinking proteins, results in greater mobility within the axonal microtubule array. Interestingly, microtubule polarity flaws arose in the axons of hippocampal neurons but not sympathetic neurons, possibly due to greater activity of the motor that opposes cytoplasmic dynein in hippocampal neurons. Consistent with that motor being kinesin-1, we found that pharmacologic hyperactivation of kinesin-1-based transport of microtubules (in the axons of both types of neurons) created microtubule polarity flaws and aberrant sliding of even very long microtubules. These data support our computational model and implicate TRIM46, PRC1 and kinesin-1 as participants in the model. In addition, the present observations challenge expectations that PRC1 and kinesin-1 would preferentially crosslink and slide, respectively, oppositely oriented microtubules as opposed to commonly oriented microtubules.

P1148

**Golgi-derived Microtubules Support Trafficking of Cargo into and out of the Golgi Apparatus**

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Microtubules (MT) are polymers that play crucial roles in cell function and survival. How MT networks are organized informs their function. In many mammalian cell types, a substantial subset of MTs is
nucleated at the Golgi during interphase. These Golgi-derived MTs (GDMTs) support cell migration and motility, post-Golgi trafficking, and maintenance of the Golgi Apparatus. The exact function of GDMTs in cargo trafficking into and out of the Golgi has not been extensively studied. Moreover, how GDMTs are nucleated, stabilized, and anchored at the Golgi membrane is not fully understood. Here, we investigate the role of MT networks on cargo trafficking into and out of the Golgi. First, we show that MTs support trafficking of cargo from the ER to the Golgi and out of the Golgi. Through colocalization analyses, we find that an inducible cargo marker travels through ER exit sites (ERES), the ER-Golgi Intermediate Compartment (ERGIC), and Golgi subcompartments before packaging into vesicles for secretion. In the absence of MTs, we find that cargo is retained in TGN subcompartments, suggesting that secretion from the Golgi itself relies on MTs. To further investigate the mechanisms that underlie this MT-based cargo trafficking, we study the roles of CLASPs. CLASP1 and CLASP2 are MT-stabilizing proteins that are important for GDMT nucleation. CLASP2 localizes to MT plus ends and the Golgi. We find that CLASP2, but not CLASP1, is required for anchoring of GDMTs. Upon depletion of CLASP2 cargo transport into the Golgi is delayed, as is cargo export from the Golgi. Thus, the GDMT subset supports cargo trafficking into and out of the Golgi. Additionally, these GDMTs must be anchored properly at the Golgi by CLASP2 to support this process. In summary, this work shows cargo transport into and out of the Golgi requires MTs. Indeed, we show that cargo exit from the Golgi relies on MTs. In addition, we find that the GDMT subset is specifically required for cargo transport into and out of the Golgi, and that this subset requires CLASP2 for anchoring at the Golgi membrane. Understanding the mechanisms of how cargo recognizes and utilizes this MT subset will provide greater insight in the function of different MT networks and their organization in interphase.

P1149

**Novel roles of microtubule networks in neuronal ageing**

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During ageing, neuronal networks face damaging insults causing their deterioration, eventually affecting cognition and mobility in the elderly. The cell biology underlying neuronal ageing is little understood. To bridge this important gap in our understanding, we have developed a quantitative *in vivo* model based on identified neurons in the adult Drosophila brain. This model reproduces hallmarks of physiological ageing known from primates: (A) axonal atrophy including axonal swellings, (B) decreased microtubule density, (C) protein aggregation and (D) synaptic alterations. Here we will present our most recent discoveries identifying a cascade of events that cause the atrophy of ageing neurons through oxidative stress leading to microtubule deregulation as a key step of axon decay. At the molecular level, we uncovered the functional requirements of key microtubule regulators in mature axons, and how age-related alterations in microtubule regulators induce changes in the properties of axonal microtubule bundles that precipitate structural changes in axons which are characteristic of aged neurons. We propose that microtubules couple oxidative stress and neuronal dysfunction during ageing and that ROS-inflicted deregulation of microtubule-binding proteins lies at the heart of axonal and synaptic atrophy during ageing. Supported by the BBSRC and the Wellcome Trust.
MTCL2 is a new Golgi-resident microtubule-regulating protein, essential for organizing asymmetric microtubule network

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The Golgi apparatus in animal cells plays important roles in regulating asymmetric organization of non-centrosomal microtubules essential for polarized vesicle transport. Several microtubule-associated proteins mediate microtubule nucleation from, and attachment to the Golgi membrane. Previously, we demonstrated that the Golgi-associated coiled-coil protein, MTCL1, plays another regulatory roles for microtubules on the Golgi membrane: it interconnects and stabilizes microtubules through its N- and C-terminal microtubule-binding domains, respectively. Here, we report a mammalian paralog of MTCL1, named MTCL2, which shows overall homology with MTCL1 except in the N-terminal microtubule binding domain. It directly bound microtubules through the conserved C-terminal domain lacking the microtubule stabilization activity, and localized to the Golgi membrane through the N-terminal coiled-coil region. MTCL2 knockdown resulted in loss of microtubule accumulation around the Golgi ribbon without affecting acetylated tubulin signals. MTCL2 depletion also induced lateral expansion of the crescent-like Golgi ribbon morphology along the nucleus, and impaired directed cell migration observed in an in vitro wound healing assay. Considering the homo-oligomerization activity of MTCL2 via the multiple coiled-coil motifs, these results suggest that MTCL2 crosslinks and accumulates microtubules on the Golgi membrane, and thus promotes compact clustering of the Golgi stacks. Interestingly, MTCL2-depleted cells at the wound edge frequently showed detachment of the Golgi ribbon from the centrosome, and disintegration of the centrosomal and Golgi-associated microtubules. This indicates a possibility that MTCL2 plays an essential role in integrating these two discrete populations of microtubules, which is particularly important in cells showing directed migration. MTCL1 and 2 were localized on microtubules in a mutually exclusive manner. Therefore, the present results suggest that cells utilize two members of MTCL protein family to differentially regulate the Golgi-associated microtubules essential for controlling cell polarity.

The microtubule minus end-binding protein CAMSAP2 organizes the microtubule network of pancreatic β-cells and is required for insulin vesicle trafficking and secretion

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The cytoskeleton microtubules are required for trafficking of secretory vesicles. Pancreatic islet β-cells have a dense and intertwined network of microtubules, which are mainly derived from the Golgi apparatus and do not have clear directionality. We recently reported that the regulation of microtubule dynamics in β-cells is coupled to glucose stimulation and it is critical for glucose-stimulated insulin secretion. However, how β-cells organize and maintain the dense microtubule network to facilitate insulin vesicle trafficking and secretion is not clear. CAMSAP2 is a microtubule minus-end binding protein, which stabilizes and positions microtubules in cells. In the mouse insulinoma MIN6 cells, CAMSAP2 forms small stretches at microtubule minus ends in the cytoplasm. Surprisingly, we found that CAMSAP2 in primary islet β-cells do not form stretches in the cytoplasm but instead are enriched at the Golgi. This unique localization of CAMSAP2 is specific to β-cells but not in other islet endocrine cells and
CAMSAP2 is independent of microtubule-binding. Depletion of CAMSAP2 by shRNA leads to disorganized microtubule network, including uneven distribution and abnormal bundling, in islet β-cells and defective insulin secretion. Biochemical analyses suggest that CAMSAP2 in islet β-cells is alternatively spliced into an isoform that is smaller than the reported full-length CAMSAP2. We proposed that this differential isoform may confer the distinct subcellular localization at the Golgi and is essential for organizing the microtubules network and supporting insulin secretion in islet β-cells.

P1152

**A new concept explaining the cell biology of axons and axon pathology**

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To develop remedial strategies for neurodegeneration, we need to improve our understanding of the cell biology of neurons - in particular their axons. Axons are the cable-like, up-to-meter long processes of neurons that wire our nervous system, and are key target sites for degenerative processes. Key roles are played by the axonal cytoskeleton (J Cell Biol 10.1083/jcb.201912081). Based on our studies of over 50 actin- and microtubule (MT)-regulators and incorporating the wider literature (Neural Dev 10.1186/s13064-019-0134-0), we propose the "dependency cycle of local axon homeostasis" as a promising new concept explaining long-term maintenance of axons and the many causes for their pathologies (animated summary: tinyurl.com/y4bd42yd). In a nutshell: (1) cargo transport is essential for axon function and physiology; (2) axonal transport requires MT bundles as essential highways, but also (3) imposes mechanical stress that damages MT bundles; (4) consequently, MT bundles have to be actively maintained; (5) this maintenance depends on materials, components and physiology supplied by transport - thus closing the dependency cycle. Breaking this cycle at any point leads to bundle decay and can explain why trauma, intoxication, various genetic defects or ageing can cause very similar pathologies. Here I will explain genes and mechanisms underpinning this model. Thus, we uncovered a number of mechanisms mediating long-term maintenance of bundled MT arrangements, including roles (a) of spectraplakins in guiding polymerising MTs, (b) of cortical collapse factors in eliminating off-track MTs leaving the bundles, (c) of MT polymerisation machinery in maintaining MT volume and bundle organisation, and (d) of the actin cortex in upholding MT polymerisation. In support of the notion that MT maintenance is dependent on axon physiology, I will demonstrate that loss of kinesin-1 and -3 cause severe MT bundle aberration through patho-mechanisms that involve harmful ROS production.

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Chaperones, Protein Folding, and Quality Control: Unfolded Protein Response

P1153

**Unexpected pathways of cell stress response to procollagen misfolding in osteoblasts**

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Typically, protein misfolding in the Endoplasmic Reticulum (ER) activates unfolded protein response (UPR) pathways via IRE1, ATF6 and PERK ER stress receptors, e.g. resulting in increased eIF2α phosphorylation followed by upregulation of ATF4 and BiP/HSPA5. However, type I procollagen
misfolding in osteoblasts often disrupts ER without upregulating BiP and ATF4, suggesting noncanonical cell stress response. We previously demonstrated such an effect in a G610C mouse model, in which a Gly610 to Cys substitution in the triple helical region of the α2(I) chain causes a moderately severe osteogenesis imperfecta. While we observed osteoblast ER disruption by this mutation, higher eIF2α phosphorylation and increased CHOP expression, we found no evidence of activation of the canonical UPR pathways. To understand the effects of this mutation, we performed single-cell RNASeq analysis of E18.5 embryonic bones from wild type, heterozygous and homozygous G610C animals, which revealed significantly altered gene expression in hypertrophic chondrocytes and mature osteoblasts. We confirmed our earlier qPCR findings of CHOP upregulation without canonical unfolded protein response (UPR). Instead of increased Hspa5 and Atf4 transcription expected in UPR, scRNASeq revealed upregulation of their paralogs Hspa9 and Atf5 in mature osteoblasts, indicating activation of unexpected cell stress pathways, which we are currently investigating. We confirmed these observations by semi-quantitative in situ mRNA analysis in fixed tissues from E18.5 embryos and 2-month-old mice. Most changes in mRNA transcripts observed by scRNASeq involved genes regulating different aspects of cellular homeostasis and osteoblast function. However, we also observed significant changes in transcription of some extracellular matrix proteins, e.g. decorin and several types of collagen. Overall, our findings not only confirm a noncanonical cell stress response to procollagen misfolding in osteoblasts but also reveal unexpected pathways of this response.

P1154

The interdomain helix between the kinase and RNase domains of IRE1 transmits the conformational change that underlies ER stress-induced activation.

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The unfolded protein response (UPR) plays an evolutionarily conserved role in homeostasis, and its dysregulation often leads to human disease, including diabetes and cancer. IRE1α is a major transducer that conveys endoplasmic reticulum (ER) stress to biochemical signals, yet major gaps persist in our understanding of how the detection of stress is converted to one of several molecular outcomes. It is known that upon sensing unfolded proteins via its ER luminal domain, IRE1α dimerizes and oligomerizes (often visualized as clustering), and then trans-autophosphorylates. The IRE1α kinase activity is required for activation of its RNase effector domain and for clustering of IRE1α. It is not yet clear if IRE1α clustering is a platform for the RNase activity, or if the two represent distinct biological functions. Here, we uncover a previously unrecognized role for helix αK between IRE1α kinase and RNase domains in conveying critical conformational changes. Using mutants within this inter-domain helix, we show for the first time that: 1) distinct substitutions (specifically, of Leu827) selectively affect oligomerization, RNase activity, and, unexpectedly, the kinase activity of IRE1α; 2) RNase activation can be uncoupled from IRE1α oligomerization, and phosphorylation of S729 marks the former but not the latter; 3) The nature of residue 827 determines the conformation that the IRE1α protein adopts, leading to different patterns of biochemical activities. In summary, this work reveals a previously unappreciated role for the inter-domain helix as a pivotal conduit for attaining the stress-responsive conformation of IRE1α.
**P1155**

**Hsp104/70 bi-chaperone system readily restores heat-induced Pab1 condensates to functional units relative to heat-denatured aggregates**

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The standard model for the heat shock response in budding yeast depends on accumulation of “abnormal” proteins and their restoration by molecular chaperones. This model assumes the abnormal proteins to be heat-damaged or misfolded mature proteins, but no specific endogenous proteins undergoing heat-induced misfolding during sublethal heat shock have yet been identified. Heat-induced misfolded proteins can be restored to functionality by molecular chaperones, but with surprisingly low efficiency. In contrast, multiple mature endogenous proteins have recently been found to form biomolecular condensates in response to heat shock, in some cases with adaptive benefits. These condensates depend on molecular chaperones for timely dispersal, raising the possibility that they are a major endogenous substrate of the chaperone machinery. Here, using a fully reconstituted system, we demonstrate that molecular chaperones efficiently disperse heat-induced condensates of endogenous poly(A)-binding protein (Pab1) in yeast. We find that heat-induced Pab1 condensates exhibit different chaperone dependence from heat-induced misfolded proteins for dispersal in two critical ways. Unlike misfolded proteins, Pab1 condensates do not require small heat shock proteins for efficient dispersal. Also, the Hsp40/J-proteins Sis1 and Ydj1 show synergistic activity with misfolded proteins as previously reported, but Pab1 dispersal depends only on Sis1 and does not induce Sis1/Ydj1 synergy. Dispersal rate and yield are markedly higher for Pab1 condensates compared to misfolded proteins. Using data from both biochemistry and kinetic simulation, we propose that this efficiency difference arises because the molecular problem of condensate dispersal differs in critical ways from the disaggregation of misfolded proteins. We propose a revised heat shock model in which heat triggers condensation of a specific set of mature proteins and molecular chaperones disperse the condensates as part of the adaptive cellular response.

**P1156**

**Characterizing the biological function of heat-induced condensation of enzyme Apa1**

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Cells have evolved to respond to external stresses such as heat. Heat shock triggers a set of conserved molecular programs including 1) a shift in translation from housekeeping genes to heat shock genes and 2) coalescence of a specific set of proteins into biomolecular condensates. We previously identified an enzyme diadenosine tetraphosphate phosphorlyase 1 (Apa1) as one of the proteins that condenses during heat shock in budding yeast. Soluble Apa1 breaks down diadenosine tetraphosphate (Ap4A) into ATP and ADP. Unlike most genes encoding condensing proteins, Apa1 is transcriptionally upregulated in response to heat. This suggests that condensed Apa1 may behave differently than soluble Apa1, and we predict condensed Apa1 may be useful to the stressed cell. We investigate what drives Apa1 to form heat-induced condensates, whether condensation affects Apa1’s enzymatic activity, and how Apa1 condensation contributes to the heat shock response. We have successfully reconstituted the Apa1 system in vitro and can manipulate both condensation and enzymatic activity. We find that Apa1
condensation behavior is both pH- and temperature-sensitive. We also find that condensation behavior is sensitive to the presence or absence of substrate. Apa1 has a paralog, Apa2, which shares 60% sequence homology with Apa1 but does not form biomolecular condensates during heat stress. These results raise questions about the sequence determinants of biomolecular condensation and also set up a system to interrogate how biomolecular condensation may be a mechanism of enzymatic regulation during heat shock.

P1157

Small heat shock protein suppresses apoptosis caused by endoplasmic reticulum stress in L6 myocytes
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In the elderly, an age-related increase in endoplasmic reticulum stress (ER stress) causes impaired muscle protein synthesis, leading to muscle atrophy. Small heat shock proteins (HSPBs) function as molecular chaperones against cellular stress. In this study, we investigated the ER stress suppression effects of HSPBs in muscle cells. The HSPB1 and HSPB5 isoforms increased protein expression in L6 cells along with muscle differentiation. Proteasome inhibition of L6 cells by MG132 treatment induced apoptosis in most cells within 24 h. Expression of HSPB1 and HSPB5 was induced in MG132-treated L6 cells, and these proteins accumulated in the ER region of the cells. Overexpression of HSPB5 via DNA transfection suppressed apoptosis induced by MG132 treatment, and knockdown of HSPB5 by siRNA promoted activation of caspase-3 induced by MG132 treatment. We identified PAK2 as a novel HSPB binding protein using the BioID method. PAK2 is a serine-threonine kinase that functions as an effector molecule of Rac1. HSPB5 was co-localized with PAK2 in intracellular vesicles in MG132-treated L6 cells. Inhibition of PAK2 activity by treatment with a PAK inhibitor significantly inhibited the phosphorylation of HSPB5, which is involved in chaperone activity. These results suggest a new regulatory mechanism involving PAK2 in the suppression of ER stress-induced apoptosis via HSPB5.

Innovative Approaches to Study Development

P1158

An active gel model of the actomyosin cortex reproduces experimental furrowed cell morphologies

The actomyosin cortex is central to many fundamental processes such as cytokinesis and migration. During cytokinesis and tissue morphogenesis, cell cortices actively exert forces to reshape cells. Previously, spatially periodic bands of actin have been observed that colocalize with furrows of the apical membrane in C elegans embryos and Drosophila tracheal tubules (Priess and Hirsh, 1986; Hannezo et al., 2015). Thus, it appears that individual cells can exhibit stable shapes with spatially modulated plasma membrane height that correlates with spatially modulated actin density. Mathematical models have shown that the active, contractile nature of the cortex can explain an inherent tendency for actomyosin structures to aggregate and self-assemble into patterns, but have not
explained how stable, persistent morphological changes can be induced by cortical forces. Here, we use an active gel model of the cortex to study the mechanisms by which the cortex can use mechanical force to spontaneously induce patterned morphological changes. We find that the cortex spontaneously assembles into spatial patterns that drive correspondingly patterned deformations in the plasma membrane. The model includes the effects of contractile active stress, viscous and frictional dissipation, diffusion, and turnover on cortical flow and dynamics. Such models have been shown to reproduce contractile instability and pattern formation of actomyosin media (Hannezo et al., 2015; Moore et al., 2014). We apply this model to a 2D planar cortex and to a tubular cortex of a given volume. In both cases, we use linear stability analysis to determine the conditions under which the cortex forms patterns and morphological changes. Using numerical simulations, we predict the patterns and shape transitions created by the cortex, finding good agreement between the two approaches. On planar membranes, we find that with slow turnover or large contractility, the actomyosin material spontaneously aggregates and forms patterns such as spots or stripes or a network-like pattern not previously observed. However, no shape changes occur. By contrast, on tubular membranes, we find that the cortex drives significant deformations of the membrane, while spontaneously forming stripes around the axis of the cylinder, provided turnover is slow and the cylinder length is less than a threshold. Regions of high density of actomyosin material exerted large centripetal contractile forces, creating furrows in the cylindrical plasma membranes reminiscent of the cleavage furrow during cell division and spatially periodic furrows in the Drosophila tracheal tube (Hannezo et al., 2015).

P1159

Cell-size differential drives aberrant clone dispersal in epithelial tissue

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How animal cells of diverse shapes and sizes pack to form functional tissue is a fundamental question in biology. Nevertheless, the precise role of differential cell shape in tissue organization and homeostasis is limited. My presentation will focus on the physical and biological basis of how cells of divergent morphologies self-organize and form epithelial tissue. As epithelial tissues develop, groups of cells related by descent tend to associate in clonal populations rather than dispersing within the cell layer. While this is frequently assumed to be a result of differential adhesion, precise mechanisms controlling clonal cohesiveness remain unknown. We employ computational simulations to modulate epithelial cell size in silico and show that junctions between small cells frequently collapse, resulting in clone cell dispersal among larger neighbors. Consistent with similar dynamics in vivo, we further demonstrate that mosaic disruption of Drosophila Tor generates small cells and results in aberrant clone dispersal in developing wing disc epithelia. We propose a geometric basis for this phenomenon, supported in part by the observation that soap-foam cells exhibit similar size-dependent junctional rearrangements. Combined, these results establish a link between cell-size pleomorphism and the control of epithelial cell packing, with potential implications for understanding tumor cell dispersal in human disease.
Epithelial tissues are the most common tissue type. Most epithelial tissues comprise of a layer one cell thick and while diverse in function, they all provide a barrier between tissues and their environments. These tissues undergo many cellular processes such as division and rearrangements, all while ensuring their integrity is uncompromised. Understanding how epithelial tissues develop into layers and maintain organization is essential in order to ascertain what is disrupted in disease conditions, such as cancer. Madin Darby Canine Kidney (MDCK) cells can form a polarized epithelial layer in culture and therefore provide a good model system for understanding monolayer development in culture. We and others have observed that these layers are frequently characterized by extralayer cells, positioned above (apical to) the layer. One possible origin for extralayer cells is an initial failure in monolayer formation, meaning that cells “clumped” on top of each other during plating. Another possibility is that newly born division products fail to integrate into the extant layer. A failure for cells to reintegrate after division would increase layer disorganization, a characteristic seen in carcinomas. To investigate the aetiology of misplaced cells, we have developed an analysis pipeline called Automated Layer Analysis (ALAn), to quantify aspects of MDCK cell layer architecture. Using this tool we can automate quantification of layer characteristics, such as density, layer height and the number of extralayer cells, making our analysis unbiased and more precise. Having verified our tool is accurate by comparing automated vs manual counts in control conditions, we are now using ALAn to look at how different variables, such as cell division and adhesion, contribute to layer development using a combination of drugs and shRNAs.

A combined chemical and mechanical signaling mechanism regulates cell division plane orientation to maintain structure of the shoot apical meristem

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One of the central problems in animal and plant developmental biology is deciphering how chemical and mechanical signals interact at the cellular and tissue level to regulate cell behaviors within a tissue and produce the final shape, size and function of an organ. In particular, how local rules controlling cell division plane orientation in the shoot apical meristems (SAMs) of plants scale to produce emergent tissue-level properties is not well understood. Recent experiments by our group reveal that the homeodomain transcription factor WUSCHEL (WUS) and the plant growth hormone cytokinin (CK) alter cell division rates and cell division plane orientation leading to distinct changes in the structural organization and overall tissue shape of the SAM. Specifically, we observe that WUS inhibits periclinal division while CK promotes it. In addition, the unique biophysical constraints imposed by the plant cell wall are thought to result in local mechanical signals impacting the choice of cell division plane orientation. To investigate the roles of chemical and mechanical signals in controlling cell division plane orientation to maintain proper shape and structural organization of the SAM we develop a novel, multiscale computational model of SAM growth. Our model includes several biologically relevant
components such as a detailed representation of the mechanics of the cell wall and experimentally calibrated individual cell concentrations of chemical signals. We use our model to investigate the impact of three mechanisms hypothesized to determine cell division plane orientation including Errera’s rule, chemical concentration dependent division plane orientation and division plane orientation perpendicular to the plane of maximum tensile stress on the cell wall. In particular, we use different metrics to compare structural organization and shape of SAM tissues between simulations employing different division plane rules and experiments to quantify the impact of each mechanism. Our results suggest that a combined chemical and mechanical signaling mechanism where high CK signaling overrides mechanical cues to promote periclinal divisions and high WUS signaling causes cells to ignore chemical signals and instead divide to alleviate tensile stress on the cell wall results in proper structural organization of the SAM observed in experiments.

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Mapping tissue properties in developing embryos employing single migrating cells as bioprobes

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Interactions among cells and tissues determine the distribution and shape of structures within the developing embryo. In general, such patterning events depend on biochemical signaling cascades and on biophysical properties of tissues. Here we report on a novel methodology for identifying tissue properties relevant for morphogenesis, employing Primordial Germ Cells (PGCs) as “bioprobes” for tissue features. Using a software platform we developed, we generated maps of cell distribution derived from a large number of embryos within which PGCs lacking the guidance receptor Cxcr4b migrate non-directionally. This analysis pipeline allowed us to identify differences among domains within the embryo that are relevant for single-cell migration. Specifically, we identified medial tissue structures that exclude the single migrating cells. We attributed this distribution to deflection of the cells from tissue borders and analyzed the border between the notochord and somitic mesoderm in more detail. We find that this border is characterized by the presence of structured ECM and differences in the tissues’ viscoelastic properties. Furthermore, we show that this methodology is also useful for analyzing the effect of mutations on tissue patterning events and can be used in embryos other than those of zebrafish.
**P1163**

**Uncovering the cell biology of ultrathin alveolar type 1 cells**  
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**Objective:** The lung epithelium is home to one of the thinnest cells in the mammalian body – alveolar type 1 (AT1) cells. Individual AT1 cells are >10,000 um² in surface area but merely 0.1 um thick, approaching the diffraction limit of light microscopy. Nevertheless, such ultrathin morphology is essential for gases to passively diffuse across into the underlying vasculature. Furthermore, these AT1 cells are active in signaling and must be mechanically strong to withstand the cyclic breathing motion, requiring their subcellular machinery to function within a confined space and over a large distance, reminiscent of axonal transport in neurons. It is unclear how the cellular components, such as cytoskeletons, organelles, and junctions, drive and adapt to the unique AT1 cell morphology, but we hypothesize that this shape change occurs via simultaneous lateral contraction and apical/basal expansion.  

**Methods:** We are pursuing this hypothesis using expansion microscopy and a novel Cre-dependent cell biology reporter mouse that marks 6 cellular components with distinct colors and is also amenable to real-time imaging. Our construct utilizes viral 2A sequences and non-overlapping fluorophores to highlight subcellular dynamics during AT1 cell morphogenesis. We also use embryonic lung explant culture to test the effect of cytoskeletal drugs on AT1 cell flattening during development.  

**Results:** We have established a robust in vivo reporter and are currently breeding mice to create a stable subcellular reporter line.  

**Conclusions:** Our work is expected to reveal untapped cell biology unique to large, thin cells and also introduce a general approach to bridge our vast knowledge of in vitro cell biology to its in vivo counterpart.

**P1164**

**Live cell imaging and snapshot measurements quantify landscape and path of mesenchymal to epithelial transition**  
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Cell phenotypic transition accompanies all developmental processes, and it is an active topic how to study the step-by-step dynamics of such transition processes. Epithelial-to-mesenchymal transition (EMT) and the reverse process mesenchymal-to-epithelial transition (MET) have been shown to be fundamental processes in developmental and physiological processes, and be involved in processes such as cancer metastasis and fibrosis progression. Investigating how EMT and MET proceed and are regulated provides deep insight on both basic mechanistic understanding and biomedical applications. Recently we developed a live cell imaging platform and identified two parallel paths for TGF-beta induced EMT in A549 cells (Wang et al. Sci. Adv., in press). In this project we aim to apply the platform to examine whether the MET process also proceeds with multiple paths and whether the EMT and MET paths are different as theoretical studies predict. We are collecting live cell imaging data, and in the process of performing fixed-cell based immuno-staining studies of selected proteins at different time points of the process. Meanwhile we have further optimized our computational package of performing deep-learning based image analysis tool package. At the time of the conference we expect to present
systematic studies on the MET processes after removing TGF-beta treatment from A549 cells, which will address the questions raised above.

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**A Genome-Wide Functional Genomics Screen for Regulators of Adipogenesis and Lipogenesis**

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The prevalence of obesity, characterized by an expansion of white adipose tissue due to excess nutrients, has reached pandemic levels. White adipose tissue expands by both generating new adipocytes and by upregulating lipogenesis in existing adipocytes. We performed a CRISPR-based, genome-wide screen to identify regulators of adipogenesis and lipogenesis in the mouse 3T3-L1 cell line model. We utilized a pooled screening strategy and fluorescence-activated cell sorting to isolate populations based on lipid content, gating for fluorescence intensity of the lipophilic, green fluorescent BODIPY 493/503 dye. Isolated cell populations were sequenced to quantify sgRNA abundance, and genes regulating adipogenesis were identified by comparing sgRNA abundance between the most differentiated cell population, defined as having a high lipid content, and undifferentiated cells. Specifically, gene targets that are required for adipogenesis would show sgRNA depletion in the most highly differentiated cells, while gene targets that normally inhibit adipogenesis would show an enrichment for a particular sgRNA. This strategy identified known and novel adipogenic regulators, including numerous genes linked to metabolic disorders in GWAS. Further, the screen defined a core set of adipogenic subprograms regulating specific steps in adipogenesis, such as mitotic expansion and lipogenesis. In particular, post-transcriptional mechanisms regulating protein abundance, including selective translation of transcripts and protein degradation by the ubiquitin proteasome pathway, were found to be critical during adipogenesis. Additional proteomic and genomic analyses confirmed that a significant fraction of changes in protein abundance during 3T3-L1 adipogenesis were not driven by transcriptional mechanisms. We exemplify these themes by demonstrating that protein hypusination, a regulator of translation, is critical for translating adipogenic inducers of mitotic expansion and that neddylation of cullin-ring E3 ubiquitin ligases modulates insulin sensitivity and regulates lipogenesis in both preadipocytes and hepatocytes. These data provide a powerful resource to the broader adipose tissue and lipid droplets research communities.

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**Sea robins as a model for evolutionary trait gain in vertebrates**

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Studies in evolutionary biology have been revolutionized by the ability to test hypotheses using modern genetics and genomics. Experiments utilizing diverse model and non-model organisms have shown that evolutionary traits can sometimes be traced to key, developmental loci that have large effects on phenotypic variation. However, the best understood examples of evolutionary change in wild organisms
have primarily focused on traits that have been lost or on the addition of serially homologous structures such as vertebrae or teeth. Less understood are the molecular mechanisms involved in the development of gained traits with novel functions. Bony fish exhibit a wide array of phenotypes adapted to different underwater environments, including variation in color, body size and shape, and fin morphology. Multiple clades of benthic dwelling fish have evolved modified pectoral and pelvic fins rendering them better adapted for life on the ocean floor. Sea robins (*Prionotus carolinus*) are one such example, exhibiting expanded pectoral fins and modified free fin rays that are separated from the pectoral fin and display unique sensory and locomotor capabilities. Additionally, this particular species has evolved elaborations of the dorsal horn of the spinal cord, termed accessory lobes, which are involved in coordinated control of the free fin rays. In order to develop sea robins as a genetic model system that can be used to study trait gain, we have sequenced the genome of a common species available near Woods Hole, Massachusetts, set up successful *in vitro* fertilization and culture conditions at the Marine Biological Laboratory and Roger Williams University, and established methods for microinjection and CRISPR-Cas9 editing of the genome. We are currently using RNA sequencing to understand the molecular basis of the fin and lobe structures. Future experiments will involve testing the biological significance of differentially expressed genes using CRISPR-Cas9 and single-cell RNA sequencing to further delineate cell type specificity and gene networks involved in fin and lobe development. Using sea robins as a model, we hope to understand whether some of the important developmental genes previously shown to play a role in evolution are also involved in the evolution of gained traits.

**P1167**

**The Allen Cell Collection: High quality, fluorescently tagged human iPS cell lines to illuminate cell and nuclear organization in differentiation, disease, and regeneration**


The Allen Institute for Cell Science has created a collection of fluorescent protein-tagged hiPS cell lines to illuminate cell organization and enable observation of cellular behavior from a structural perspective. To date, the Allen Cell Collection consists of >40 single-, dual-, or triple-edited lines that have undergone extensive quality control testing to ensure genomic, cell biological, and stem cell integrity. We have tagged the major cellular organelles, a few signaling molecules, membrane-less and cardiomyocyte-specific structures, phase transition markers, and transcription factors. Our most recently released lines include mitochondrial transcription factor A (TFAM), triple-edited fibrillarin/nucleophosmin 1/citramalyl-CoA lyase safe harbor (FBL/NPM1/CLYBL-dCas9-KRAB), triple-edited fibrillarin/nucleophosmin 1/nucleolar transcription factor UBF (FBL/NPM1/UBTF), a DNA and RNA binding protein (SON), the nuclear envelope (LMNB1-mTagRFP-T), a polycomb repressor complex (EZH2), RNA polymerase (POLR2A), and dystrophin (DMD), a cardiomyocyte and skeletal muscle-specific gene. We have also released a dCas9 line (dCas9-KRAB) which enables CRISPRi-mediated knockdown of target genes. Here, we present our gene-editing and quality control workflows for mono- and biallelic editing of expressed or silent genes that are expressed during differentiation. We also highlight the utility of our CRISPR interference hiPSC lines, which express dCas9-KRAB, in a proof-of-concept, FACS-based, pooled genetic screen to identify genes associated with perturbed nucleolar structure. Furthermore, we will discuss two of our applications of the Allen Cell Collection; we use high-replicate, high-resolution, 3D live images of
our cell lines as well as quantitative analysis and modeling approaches to create integrative cell models such as 1) the Integrated Mitotic Stem Cell and 2) the Integrated Nucleus. The first captures a holistic view of the human cell division using 15 key cellular structures, and the latter will conjoin key nuclear organizational landmarks with chromatin architecture in collaboration with the 4D Nucleome Project. Our cell lines, the plasmids used to generate them, thousands of segmented single cell 3D images of our lines, analysis and visualization tools, integrated cell models and biological findings are freely available to the research community (www.allencell.org).

Kinesin Regulation

P1168

**Phosphorylation of KIF18A alters its mitotic function and localization**

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Kinesin motor proteins play important roles in intracellular transport, cell division, and cilia function. Eukaryotic cells express many kinesins (45 in mammals) to support these diverse cellular processes in cell cycle and cell type dependent manners. Of the 45 mammalian kinesins, 44 of them have identified sites of post-translational modification (PTM) within their enzymatic region, suggesting the activity of kinesins may be reversibly regulated. Our long-term goal is to investigate how kinesin function is controlled through these PTMs. We are currently investigating the effects of three previously identified phosphorylation sites (S244, S284, and S357) on the mitotic functions of KIF18A. KIF18A (kinesin-8) is a mammalian kinesin that suppresses the dynamics of microtubules to promote chromosome alignment during cell division. KIF18A is also known to play a role in kinetochore microtubule attachment and the metaphase to anaphase transition in some cell types. However, little is understood about how KIF18A is regulated to perform these various functions. Our data suggest that phosphorylation at S357 promotes the localization of KIF18A to non-kinetochore microtubules within the spindle. Additionally, mutation of S284 leads to accumulation of KIF18A at centrosomes, while a non-phosphorylatable substitution at S244 increases the time required to complete mitosis. Taken together this work indicates that phosphorylation of residues within KIF18A’s enzymatic region are important for controlling its activity and localization during cell division.

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**The tail of kinesin-14a in Giardia is a dual regulator of motility**

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Kinesin-14s are microtubule-based motor proteins that play important roles in mitotic spindle assembly. Ncd-type kinesin-14s are a subset of kinesin-14 motors that exist as homodimers with an N-terminal microtubule-binding tail, a coiled-coil central stalk (central stalk), a neck, and two identical C-terminal motor domains. To date, no Ncd-type kinesin-14 has been found to naturally exhibit long-distance minus-end-directed processive motility on single microtubules as individual homodimers. Here, we show
that GiKIN14a from \textit{Giardia intestinalis} is an unconventional Ncd-type kinesin-14 that uses its N-terminal microtubule-binding tail to achieve minus-end-directed processivity on single microtubules over \textmu{}m distances as a homodimer. We further find that while truncation of the N-terminal tail greatly reduces GiKIN14a processivity, the resulting tailless construct GiKIN14a-\textDelta{}tail is still a minimally processive motor and moves its center-of-mass via discrete 8-nm steps on the microtubule. In addition, full-length GiKIN14a has significantly higher stepping and ATP hydrolysis rates than does GiKIN14a-\textDelta{}tail. Inserting a flexible polypeptide linker into the central stalk of full-length GiKIN14a nearly reduces its ATP hydrolysis rate to that of GiKIN14a-\textDelta{}tail. Collectively, our results reveal that the N-terminal tail of GiKIN14a is a \textit{de facto} dual regulator of motility and reinforce the notion of the central stalk as a key mechanical determinant of kinesin-14 motility.

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\textbf{Distinct Tubulin Mutations Cause Similar Neurological Diseases by Inhibiting or Hyper-activating Kinesin Activity}

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The microtubule cytoskeleton and kinesin-mediated microtubule-dependent transport are essential for nervous system development and homeostasis. Congenital fibrosis of the extraocular muscles (CFEOM) is an autosomal dominant disorder characterized by failed axon growth and/or maintenance of the oculomotor neuron resulting in drooping eyelids with limited eye movements. Mutations in the kinesin KIF21A cause isolated CFEOM, while those in the beta-tubulins TUBB3 or TUBB2B cause CFEOM accompanied by a spectrum of additional axon-related malformations ranging from mild to severe. It was previously shown that, although CFEOM-causing mutations in KIF21A do not alter the run-length or velocity, they increase the binding of the mutant motors to microtubules, suggesting these mutations disrupt oculomotor neuron development by hyper-activating KIF21A. Our previous work indicates that CFEOM mutations in TUBB3 or TUBB2B generally diminish kinesin binding and suggest they cause disease by decreasing kinesin-dependent activity. We utilized a Drosophila S2 cell-based assay to quantify kinesin-dependent vesicle transport in the presence and absence of mutant human TUBB3. Our results demonstrate that, relative to wildtype TUBB3, CFEOM mutations inhibit anterograde vesicle movements and decrease transport distance. Consistent with the idea that these defects underlie axon disorder, TUBB3 mutations causing cortical malformations independent of CFEOM do not inhibit vesicle transport. Most CFEOM mutations, including D417H, diminish kinesin localization to microtubules when modeled in yeast tubulin. We found the R380C substitution, however, significantly increases localization in yeast cells. We utilized total internal reflection fluorescence (TIRF) microscopy to reconstitute KIF21A transport on wild type, R380C, and D417H microtubules purified from yeast. Compared to wild type, kinesin binding and transport were severely diminished on D417H microtubules. Strikingly, we find KIF21A binding rate, activation frequency, run length, and localization to the microtubule end are significantly increased on R380C microtubules relative to wild type. These results suggest a new paradigm for CFEOM tubulin mutations. Whereas most tubulin mutations examined to date disrupt axon growth/maintenance by decreasing the activity of KIF21A and other kinesins, mutations such as R380C produce similar disease by aberrantly increasing kinesin activities. These results show that tubulin can regulate the activity of motors that operate on the microtubule track. To understand tubulin-related disorders it will be important to discern whether disease-causing mutations can differentially affect specific subsets of kinesin motors.
Kinesin-2 ciliary entry is impaired following MAPK activation

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Ciliary length and regulation are dictated by intraflagellar transport (IFT) which transports building materials into cilia to regulate assembly. Recent research has revealed multiple molecular targets in the Mitogen Activated Protein Kinase (MAPK) pathway that regulate ciliary length in many different types of cells from photoreceptors to C. elegans sensory neuron cilia. However, the mechanism that connects how MAPK regulates this function is unclear. Previously we have found that BCI, (E)-2-benzyldiene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one, a vertebrate MAP Kinase Phosphatase 3 inhibitor, increases phosphorylated MAPK, shortens cilia, and prevents regeneration of full-length cilia in Chlamydomonas reinhardtii. Here we show that prolonged treatment with BCI alters kinesin-2 motor dynamics. Using Total Internal Reflection Microscopy (TIRF) with GFP-tagged kinesin-2 subunits, we find over the course of a 2 hour BCI treatment that the speed of kinesin-2 remains unchanged while train size and frequency decrease significantly. This decreased frequency identified with TIRF is accompanied by a gradual increase in GFP-tagged kinesin-2 fluorescence intensity at the base of the cilia. We hypothesize that this decreased IFT frequency and impaired regeneration of full-length cilia is caused by alteration of either kinesin-2 or transition zone properties as a result of MAPK activation.

Two functionally redundant sliding modules drive chromosome segregation

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Proper chromosome segregation into two future daughter cells requires the mitotic spindle to elongate in anaphase. Yet, the molecular mechanism that drives spindle elongation in human cells is unknown. Using combined depletion and inactivation assays together with CRISPR technology to explore redundancy between multiple targets, we discovered that the force-generating mechanism of spindle elongation consists of the PRC1-dependent motor KIF4A/kinesin-4 together with EG5/kinesin-5. Disruption of both motors leads to total failure of chromosome segregation due to blocked spindle elongation, despite poleward chromosome motion. Tubulin photoactivation and super-resolution microscopy show that perturbation of both proteins impairs midzone microtubule sliding without affecting microtubule stability. Thus, two mechanistically distinct sliding modules, one based on a self-sustained and the other on a crosslinker-assisted motor, power the mechanism that drives spindle elongation in human cells.

The Kinesin-14 tail contains importin α/β dependent and independent MT binding domains

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Centrosome amplification is a hallmark of cancer cells and results in the formation of multipolar spindles. If multipolar division occurs, the daughter cells die due to severe aneuploidy. Often, these cells cluster their centrosomes leading to bipolar divisions with mild aneuploidy, allowing daughter cell survival. Kinesin-14s play a prominent role in centrosome clustering, as their inhibition is lethal to cells with centrosome amplification but not to normal cells. Kinesin-14s cross-link and slide both parallel and anti-parallel microtubules (MTs), but the mechanism by which this occurs is unknown. Our recent work suggests that the RanGTP gradient promotes preferential Kinesin-14 parallel MT cross-linking at the poles where importin α/β binding to the Kinesin-14 tail inhibits anti-parallel MT cross-links. These findings suggest that the tail domain contains two independent MT binding domains (MBDs), one which would cross-link anti-parallel MTs and a second that would cross-link parallel MTs. To test these ideas, we performed a deletion analysis on the tail domain of the *Xenopus* Kinesin-14 XCTK2 and identified two independent MBDs using MT binding assays. MBD1 contained the nuclear localization signal (NLS) and interacted with importin α/β. Mutation of MBD1 in the full-length tail domain reduced MT affinity and made it insensitive to importin α/β regulation. In contrast, mutation of MBD2 in the full-length tail domain further reduced MT affinity and prevented MT binding in the presence of importin α/β. Mutation of both MBDs in the tail domain resulted in severe inhibition of MT binding. Together, these results show that the tail domain of XCTK2 contains two independent MBDs that are differentially regulated by importin α/β. To test whether MBD1 and MBD2 cross-link anti-parallel and parallel MTs, respectively, we are currently expressing full-length proteins with the different MBD mutations and will test their MT cross-linking abilities *in vitro*. We have also generated lentiviruses and plan to test the abilities of the MBD mutants to cluster centrosomes in cells. We propose that a preference in parallel MT cross-linking near the poles could be a mechanism by which cancer cells cluster their centrosomes. Identifying the mechanism by which Kinesin-14s promote centrosome clustering could thus become an important therapeutic target.

**Kinetochoore Assembly and Function**

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**Reconstitution of human kinetochoore in mitotic cell extracts reveals permitted and restricted assembly steps**

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Assembly of a functional kinetochoore and its proper interaction with spindle microtubules underlie accurate chromosome segregation. Functional kinetochores can be assembled in phosphorylation-dependent manner *in vitro* using yeast cell extracts and *Xenopus* egg extracts. Although analogous molecular interactions have been reported between individual kinetochoore components in human cells, it is still unclear whether active kinetochoore complexes can form *de novo* in extracts of mitotic human cells. By analyzing the recruitment of GFP-fused kinetochoore proteins from human mitotic cell extracts to inner kinetochoore components immobilized on the microbeads, we found that only a few assembly steps are permitted in this system. We reconstructed the interaction between CENP-C and CENP-A-containing nucleosomes. Subsequent phospho-dependent recruitment of the Mis12 complex was also
detected, however, recruitment of the Ndc80 complex via both CENP-T and CENP-C pathways was inhibited. Consistently, the microtubule-binding activity of native kinetochore components, as well as those assembled using a combination of native and recombinant proteins, was weaker than that of recombinant Ndc80 complex alone. Our results demonstrate that formation of kinetochore complexes with robust microtubule-binding activity in cell extracts is impeded by multiple mechanisms, including inhibition of the recruitment of Ndc80 and Mis12 complexes to CENP-T, partial inhibition of binding between CENP-C and the Mis12 complex, as well as inhibition of microtubule binding of native Ndc80 complex. Such inhibitory mechanisms are likely to guard against spurious formation of kinetochore complexes in the cytosol of mitotic human cells, which could compete with the formation of chromosomal kinetochores and interfere with chromosome motility, spindle function, and checkpoint signaling.

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A spatiotemporally controlled establishment of asymmetric CENP-A at sister centromeres during cell cycle of stem cells

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Many stem cells utilize asymmetric cell division (ACD) to produce a self-renewed stem cell and a differentiating daughter cell. How non-genic information could be inherited differentially to establish distinct cell fates is not well understood. Here, we report a series of spatiotemporally regulated asymmetric components, which ensure biased sister chromatid attachment and segregation during ACD of *Drosophila* male germline stem cells (GSCs). First, sister centromeres are differentially enriched with proteins involved in centromere specification and kinetochore function. Second, temporally asymmetric microtubule activities and polarized nuclear envelope breakdown allow for the preferential recognition and attachment of microtubules to asymmetric sister kinetochores and sister centromeres. Abolishment of either the asymmetric sister centromeres or the asymmetric microtubule activities results in randomized sister chromatid segregation and stem cell defects. Together, these results provide the cellular basis for partitioning epigenetically distinct sister chromatids during stem cell ACDs. However, how asymmetric sister centromeres are established with identical genetic sequence is not well understood. I performed Super-Resolution Chromatin Fiber (SRCF) assay to visualize newly replicated sister chromatids. I found that one sister chromatid inherited more CENP-A compared to the other, suggesting pre-existing (old) CENP-A is recycled asymmetrically during DNA replication. Further, the inner-kinetochore protein CENP-C shows a similar asymmetric pattern, the sister chromatid with more CENP-A shows more CENP-C than the other sister chromatid, indicating a relay of asymmetry from centromere to kinetochore. I found that the CAL1 chaperone specific for CENP-A is required for both old CENP-A recycling during S phase and new CENP-A incorporation during S/G2 phase in male GSCs. Compromising CAL1 activity abolishes both CENP-A and CENP-C asymmetry and results in mis-determination of GSC and progenitor germ cell fate, resulting in both GSC loss and early-stage germline tumor phenotype. Together, these results show the molecular mechanisms underlying the establishment of asymmetric sister centromeres in GSCs and opens new directions to study these phenomena in other biological contexts.
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Centromere drive and suppression by parallel pathways for recruiting microtubule destabilizers


Selfish centromere DNA sequences can bias their transmission to the egg in female meiosis. Evolutionary theory suggests that centromere proteins evolve to suppress such centromere drive. In mouse models, selfish centromere DNA hijacks a kinetochore pathway to recruit microtubule-destablizing proteins that act as effectors for drive. We show that drive is suppressed by minimizing the kinetochore pathway relative to a second pathway for effector recruitment by heterochromatin, which equalizes centromeres. Disruption of the kinetochore pathway by introducing a divergent allele of CENP-C reduces functional differences due to selfish centromere DNA, whereas disruption of the suppressor heterochromatin pathway by CENP-B deletion amplifies the differences. Furthermore, molecular evolution analyses using newly-sequenced Murinae genomes identify adaptive evolution in multiple protein domains that lead to effector recruitment. Together, these findings suggest that the chromosome segregation machinery has recurrently evolved to suppress drive by modulating parallel pathways for recruiting microtubule destabilizers while maintaining essential functions.

P1177

A non-centromeric locus experiencing female meiotic drive in mice

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A cornerstone of meiosis is equal but random segregation of homologous chromosomes, serving to produce complete haploid genomes and genetic diversity among gametes. However, some genetic elements exhibit selfish mechanisms, altering chromosome segregation for their own benefit, often at the cost of organismal fitness and fertility. Mouse chromosome 2 harbors such a genetic element, a repeat with a 127 kb monomer, called R2d2. Known to experience transmission ratio distortion (TRD) in heterozygous females, R2d2 is thought to preferentially segregate to the egg in female meiosis. This TRD is also correlated with decreased fertility. Understanding the cell biological basis of this behavior is not only informative for genome evolution, but has important implications for our knowledge of proper chromosome segregation and genetic predispositions for aneuploidy and infertility. We found that in heterozygous mice, the chromosome with the R2d2 repeat lags during anaphase of meiosis I. In female meiosis, cytokinesis is asymmetric, incorporating most of the cytoplasm into the egg. Therefore, this lagging behavior could allow R2d2 to avoid segregation to the polar body by remaining near the spindle midzone long enough to be incorporated into the egg. To better examine this, cytogenetic studies of mouse oocytes provide a tractable tool. Immunofluorescence (IF) and chromosome oligopaint technologies offer a means to track R2d2’s movement, preferential segregation frequency, recombination rates and colocalization with various proteins during meiosis I and II. These data collectively allow us to test the hypothesis that R2d2 alters its own segregation by acting as a neo-centromere.
P1178

**Mps1 promotes poleward chromosome movements in meiotic pro-metaphase**

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In prophase of meiosis I, homologous partner chromosomes pair and become connected by crossovers. Chiasmata, the connections formed between the partners enable the chromosome pair, called a bivalent, to attach as a single unit to the spindle. When the meiosis I spindle forms in prometaphase, most bivalents are associated with a single spindle pole and go through a series of oscillations on the spindle, attaching to and detaching from microtubules until the partners of the bivalent are bi-oriented, that is, attached to microtubules from opposite sides of the spindle, and prepared to be segregated at anaphase I. The conserved, kinetochore-associated kinase, Mps1, is essential for the bivalents to be pulled by microtubules across the spindle in prometaphase. Here we show that *MPS1* is not required for kinetochores to attach microtubules and is not required for normal rates of microtubule depolymerization to pull chromosomes on the spindle. Instead, we show that *MPS1* is necessary to trigger depolymerization of microtubule ends once they attach to kinetochores. Thus, Mps1 acts at the kinetochore to co-ordinate the successful attachment of a microtubule and the triggering of microtubule depolymerization to move the chromosome.

P1179

**Kn1-1 and knl-3 are required for pre-anaphase pulling forces, anaphase a and homolog separation during *C. elegans* female meiosis**

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Anaphase chromosome movement is thought to be mediated by pulling forces generated by end-on attachment of microtubules to the outer face of kinetochores through the KMN network. However, it has been suggested that during *C. elegans* female meiosis, anaphase is mediated by a kinetochore-independent pushing mechanism with microtubules only attached to the inner face of segregating chromosomes (Dumont et al. 2010. Nat Cell Bio 12:894; Laband et al. 2017. Nat Comm 8: 1499). We found that the KMN network proteins KNL-1 and KNL-3 are required for pre-anaphase chromosome stretching, suggesting a role in pulling forces. In the absence of KNL-1,3, pairs of homologous chromosomes did not separate and did not move toward a spindle pole. Instead, each homolog pair moved together with the same spindle pole during anaphase B spindle elongation. Two masses of chromatin thus ended up at opposite spindle poles, giving the appearance of successful anaphase. The KMN network protein, NDC-80, required KNL-1 and 3 for localization to kinetochores and NDC-80 was also required for pre-anaphase chromosome stretching and homolog separation. This requirement for NDC-80 was surprising because it is thought to act at end-on microtubule attachments, but only lateral microtubule contacts have been observed in these spindles by electron microscopy (Redemann et al. 2018. Curr Biol 28: 2991). Anaphase B proceeded at normal or faster than normal velocities in KNL-1,3-depleted embryos but was highly error prone as both homologs of the same bivalent moved with the same spindle pole.
Aneuploidy can occur due to persistent mono-oriented chromosomes
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Unequal chromosome segregation which causes aneuploidy can happen due to improper attachments between kinetochores and microtubules. While it was shown that lagging chromosomes can cause aneuploidic state, it has not yet been proven that the same applies for mono-oriented chromosomes. Here, we quantitatively assessed errors in chromosome segregation in 2D cultures, both in healthy (RPE1) and cancer human cell lines (U2OS and HeLa) and found that cancer cells can enter anaphase with persistent mono-oriented chromosomes. We found this rate to be 3-4% in U2OS and HeLa cells. By increasing chromosome segregation error rates with nocodazole, we also observed persistent mono-oriented chromosomes in RPE1 cells after nocodazole washout. Prior to anaphase, the frequency of mono-oriented chromosomes was roughly 2x higher in U2OS than in HeLa cells. In U2OS cells, we found a 70-minute delay in anaphase entry during which most of the mono-oriented chromosomes were resolved. While attempting to congress, some mono-oriented chromosomes underwent back-and-forth movements towards the metaphase plate multiple times. In these chromosome motions, inter-kinetochore distance increased while moving towards the metaphase plate and decreased while moving towards the pole. Interestingly, we found multiple mono-oriented chromosomes in 2% of U2OS cells. Our results reveal that mono-oriented chromosomes are an evident cause of aneuploidy, presumably due to spindle assembly checkpoint (SAC) override, and suggest that multiple errors per cell happen due to defects in the mitotic machinery.

Centromeric transcription Maintains Centromeric Cohesion in human cells
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Transcription at human centromeres containing non-coding repetitive α-satellite DNA sequences has been shown to play an important role in centromere functions. However, lack of approaches to specifically manipulate centromeric transcription makes it unclear whether the proposed functions are the direct consequences of centromeric transcription. By monitoring nascent RNAs, we found that several commonly used transcriptional inhibitors exhibited distinct, even opposing, efficacies on the suppression of ongoing gene and centromeric transcription; whereas, under the same conditions, total centromeric RNAs were changed to a lesser extent. The inhibitors suppressing ongoing centromeric transcription weakened centromeric cohesion in mitotic cells and the one increasing ongoing centromeric transcription strengthened centromeric cohesion. Furthermore, expression of CENP-B DB (DNA binding-domain) moderately increased centromeric cohesion without altering gene transcription; and as a result, centromeric cohesion was accordingly strengthened. Targeting of the transcriptional suppressor Kox1 with CENP-B DB to centromeres specifically decreased centromeric transcription and weakened centromeric cohesion in mitotic cells. Thus, based on these findings, we propose that a major function of centromeric transcription is to maintain centromeric cohesion during mitosis in human cells.
Lipid Droplets

P1181

Multiple C2 and transmembrane domain containing proteins, MCTP1 and MCTP2, are at Lipid Droplet formation sites in the ER membrane.
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Lipid Droplets (LDs) are neutral lipid-containing organelles that are enclosed in a single monolayer of phospholipids. LD formation begins in the ER membrane with accumulation of neutral lipids within the bilayer. When the amount of neutral lipid accumulation reaches a critical mass the LDs biophysically separate from the ER membrane. It is not known how LD formation sites in the ER membrane are determined; whether these sites are random or pre-determined ER subdomains. We show that multiple C2 and transmembrane domain containing proteins, MCTP1 and MCTP2, are at LD formation sites in specialized ER subdomains. MCTP proteins have three C2 domains at the N-terminus and a transmembrane domain at the C-terminus. HHpred analysis suggests that the transmembrane region of MCTPs is similar to the reticulon homology domain (RHD). Similar to reticulons, these proteins when overexpressed are able to tubulate the ER membrane and favor highly curved regions of the ER membrane, as they localize to ER tubules and edges of ER sheets. MCTP1 and MCTP2 regulate LD biogenesis, as overexpression of these proteins increases the number and size of LDs. Interestingly, overexpression of only the RHD resulted in increase in number but not size of LDs. This indicates that the MCTP RHD domain regulates LD number, while, the C2 domains are required to regulate LD size. We find that the C2 domains have the ability to bind to charged phospholipids such as PI4P, PI4,5P2, PI3,4,5P3 and cardiolipin. Based on our findings, we propose that MCTP proteins localize at discrete ER subdomains that facilitate organelle formation by generating ER membrane bending by the RHD, and organelle contact sites through the C2 domains.

P1182

Apolipoprotein E targets cytoplasmic lipid droplets in response to lipid accumulation in astrocytes
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Apolipoprotein E (ApoE) is the principal vehicle of intercellular lipid trafficking in the central nervous system. Astrocytes secrete ApoE-containing lipoprotein particles carrying cholesterol and unsaturated phospholipids. Neurons take up these lipoproteins via endocytosis and use the lipids provided by astrocytes to build vast membranes and synapses. ApoE can also transport lipids from neurons under oxidative stress and shuttle them back to astrocytes, where they are stored in cytoplasmic lipid droplets (LDs), as a neuroprotective mechanism. The importance of ApoE in the central nervous system is underlined by its association with Alzheimer’s Disease (AD). The \textit{APOE} gene is the strongest genetic risk factor for sporadic late-onset AD. Individuals with the \textit{APOE4} variant have an increased risk of developing AD later in life compared to those who possess the \textit{APOE3} variant. However, little is known about the trafficking of ApoE in astrocytes or how it impacts intracellular lipid metabolism. We have discovered that, in response to lipid accumulation, ApoE targets to the cytoplasmic surface of lipid droplets (LDs) rather than progressing through the secretory pathway. Live-cell imaging and...
fluorescence recovery after photobleaching (FRAP) experiments demonstrate that ApoE protein translocates from the ER lumen to LDs at ER-LD contact sites. The C-terminal domain of ApoE is responsible for LD binding, while the N-terminal domain is necessary for efficient targeting of ApoE to the cytosolic compartment. Mutations of positively charged residues in the N-terminal domain of ApoE, including the rare AD-protective Christchurch variant of ApoE, increase targeting to LDs. Together, these results indicate that ApoE can target LDs within astrocytes instead of being secreted and may play a role in regulating the balance between lipid secretion and intracellular lipid storage and metabolism.

P1183

The GTPase Activity of the Double FYVE Domain Containing Protein 1 is Essential for Lipid Droplet Metabolism.

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Double FYVE Domain Containing Protein 1 (DFCP1) is a common marker for autophagy and has been implicated in establishing contacts between the endoplasmic reticulum (ER) and lipid droplets (LDs), however, little is known about the protein’s function in LD dynamics, metabolism, or lipophagy. Here, we demonstrate that endogenous DFCP1 localizes to LDs independent of its FYVE domains, and that endogenous levels of DFCP1 are sufficient to control cellular fatty acid levels. Indeed, DFCP1-knockdown increases cellular free fatty acid uptake and neutral lipid content, resulting in cells that contain more LDs than control cells, and these differences are exacerbated under starved conditions. We also find that LD localization requires a combination of an ER-binding domain and a previously unidentified GTPase domain. Using spectroscopic approaches, we indicate that this GTPase domain has several distinguishing properties, including the ability to oligomerize and a low basal GTP turnover rate like that of Ras-like GTPases. Finally, we show that mutations that disrupt DFCP1’s GTPase activity alter accumulation of DFCP1 on LDs and modulate free fatty acid storage. Collectively, our findings support a mechanism whereby GTP-dependent localization of DFCP1 to LDs directly modulates cellular storage of free fatty acids via control of LD growth and degradation.

P1184

Seipin traps triacylglycerols in the ER to facilitate their nanoscale phase separation

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Seipin is a disk-like oligomeric ER protein important for lipid droplet (LD) biogenesis. We recently reported that seipin facilitates triglyceride (TAG) delivery to the LD. It is not known how this seipin-mediated TAG flux is achieved. Here we investigate this through biomolecular simulations bridged to in vivo experiments in cells. Our data indicate that seipin can trap TAGs in the ER bilayer via the luminal hydrophobic helices of the protomers delineating the inner opening of the seipin disk. This promotes the nanoscale phase separation of TAGs at a concentration that by itself is insufficient to induce TAG clustering in a lipid membrane. We identify a key residue in the α3 helix and show that mutating it compromises the ability of seipin complexes to sequester TAG in silico and to promote TAG transfer to LDs in cells. Whilst a mutant compromised in TAG sequestering interacts poorly with promethin, the binding of nascent wild-type seipin complexes to promethin is promoted by TAGs. Together, these
results suggest that seipin traps TAGs via its luminal hydrophobic helices, serving as a catalyst for seeding the TAG aggregate from dissolved monomers inside the seipin ring, thereby generating a favorable promethin binding interface.

P1185

**Mechanism of lipid droplet/mitochondria contacts and role of Perilipin 5 in lipid metabolism**

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The lipid droplet (LD) serves as a cellular lipid store; however recent work has highlighted the ability of LDs to regulate intracellular lipid trafficking. Perilipins are LD proteins which function as lipolytic barriers and protect the cell from lipotoxicity as well as excess reactive oxygen species. A recently characterized member of the family, Perilipin 5 (Plin5), is enriched in β-oxidizing tissues where it serves as a lipolytic barrier under basal conditions. Upon protein kinase A (PKA)-stimulated phosphorylation, however, Plin5 promotes lipolysis through recruitment of the lipase ATGL and its activator CGI-58. In addition to playing a role in lipolytic control, Plin5 contains a unique C-terminal domain which has been shown to promote contacts between LDs and mitochondria (Mito). However, the mechanism by which this occurs and the physiological significance of these contacts remains to be elucidated. Utilizing predictive modeling we have identified and generated constructs containing a minimal contact domain capable of inducing LD/Mito contacts. Overexpression of Plin5 lacking this contact domain (Plin5 CA) leads to accumulation of lipid droplets to the same degree as full-length Plin5, indicating that LD/Mito contacts are not required for the lipolytic barrier function of Plin5. Affinity purification-mass spectrometry utilizing Plin5 and Plin5 CA constructs indicates a role for previously unidentified protein interactors to mediate LD/Mito tethering. To assess the physiological roles of Plin5’s functional domains in fatty acid (FA) trafficking we have utilized a fluorescent FA pulse-chase assay. Our data demonstrates that efficient FA trafficking in response to nutrient deprivation is dependent on LD/Mito contacts as well as phosphorylation of Plin5 at serine 155, a known PKA phosphorylation site. Considering the expression of Plin5 primarily in β-oxidizing tissue, we hypothesize that LD/Mito contacts are not primarily involved in FA storage but rather enhance the transfer of FAs from LD to mitochondria under physiological conditions that stimulate PKA activity, leading to increased FA oxidation.

**Mitochondrial Dynamics**

P1186

**Drp1 regulates nucleoid segregation by modifying the structure of the endoplasmic reticulum**

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Mitochondria are important organelles required for multiple metabolic activities including ATP production. To function, mitochondria require proteins coded by both nuclear and mitochondrial DNA (mtDNA). Mutations in either genome lead to an array of mitochondrial diseases, with mutations in nuclear genes affecting either a specific metabolic activity or mtDNA maintenance. mtDNA is organized
into discrete nucleoprotein foci within mitochondria called nucleoids. Although the mechanism of mtDNA replication is well defined, the processes regulating segregation and distribution of nucleoids across the mitochondrial network are still unclear. Mitochondrial dynamics, the process by which mitochondria fuse and fragment to reorganize mitochondrial network in response to cues such as nutrient stress, has previously been suggested to regulate mtDNA. In fact, mitochondrial fusion is essential for mtDNA replication. On the other hand, while loss of mitochondrial fission causes nucleoid aggregation, its role in nucleoid segregation and distribution across the mitochondrial network are still unclear. Here, we show that the mitochondrial fission protein DRP1 regulates nucleoid segregation by altering the endoplasmic reticulum (ER) structure. Specifically, we found that the enlarged nucleoids present in DRP1 mutant primary human fibroblasts were spatially correlated with altered ER sheets. These cells also had an aberrant distribution of nucleoid across mitochondrial networks and enrichment of electron chain components at the site of enlarged nucleoids. Overall, our data demonstrate that mitochondrial fission control nucleoid division through regulating ER structure, which in turn is crucial for proper functioning of mitochondria.

P1187

Metabolic and dynamic heterogeneity in live single-cell mitochondria revealed via the phasor approach to FLIM

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Mitochondrial dynamics and motility have long been studied in neurons, where distal ends require a localized fuel source for their bioenergetic demands. Recent studies have elucidated the importance of mitochondrial movement to the leading edge in fueling metastasis and has sparked a new interest in the consequences of mitochondrial reorganization both spatially and bioenergetically within epithelial cells and their diseased states. We have developed a novel method to track metabolic shifts spatiotemporally within individual mitochondria in living cells. To accomplish this, we have created an unbiased and automatic mitochondrial segmentation and tracking algorithm and extended the use of the phasor approach to the fluorescence lifetime imaging (FLIM) of the intrinsically fluorescent reduced form of nicotinamide adenine dinucleotide (NADH) to observe and quantify metabolic changes within individual mitochondria in a spatiotemporal manner. Specifically, we track mitochondria using a low concentration of TMRM and analyze differences in metabolism via the fluorescence lifetime of NADH, which informs us on the free and bound states of the cofactor, which represent a glycolytic and oxidative metabolic phenotype, respectively. When analyzed in the Fourier space, we are able to quantify the fraction of free and bound NADH based on its respective position in the linear trajectory between 100% free and 100% bound NADH. We apply this approach to each individual mitochondrion, allowing us to analyze its metabolic index. We found that mitochondria that deviate from the mean metabolic index in either the oxidative or glycolytic direction have a decreased area. We also found that TMRM intensity, an indicator of bioenergetic capacity, decreased in highly oxidative mitochondria. We found also that speed decreased in highly glycolytic and highly oxidative mitochondria. Most interestingly, however, we found that only the highly oxidative mitochondria, despite its reduction in speed, are transported much more directionally, as quantified by its final displacement divided by the total distance traveled. These results hint that highly oxidative mitochondria are limited in their free NADH pools, preventing the construction of a functional electron gradient. This directionality may indicate the cell's attempt at a refueling of the mitochondrion's coenzymatic stock. To our knowledge
this is the first time that metabolic alterations in relation to mitochondrial motility have been analyzed on a single mitochondrion level in living cells.

P1188

Defining the mechanisms by which disease-associated mutations in Drp1 impede mitochondrial fission

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Mitochondria are dynamic organelles that continuously undergo fission and fusion. Fission is necessary to ensure mitochondria are properly distributed and remove damaged mitochondrial components. However, excessive fission is associated with pathogenic outcomes in cells and tissues that are especially sensitive to mitochondrial dysregulation, such as neurons. Recently, whole-exome sequencing of patients with severe dysregulation of mitochondrial dynamics has identified point mutations in dynamin-related protein 1 (Drp1), the chief regulator of mitochondrial fission. Patients present with severe neurological defects, and patient-derived fibroblasts show hyperfused mitochondria. Thus, Drp1 patient mutations impair mitochondrial fission, but it is unclear how these mutations specifically disrupt Drp1 function. As a cytosolic GTPase, Drp1 self-assembles into spirals that encircle the outer mitochondrial membrane (OMM) at sites of ensuing fission. GTP hydrolysis invokes conformational changes in Drp1 oligomers that impart mechanical force and constrict the mitochondria. The critical intra- and inter-molecular interactions that govern the oligomeric state of Drp1 and the nucleation of Drp1 polymerization on the OMM are poorly defined. Because these patient mutations are located throughout the structure of Drp1, in both the catalytic and self-assembly domains, we believe these mutations represent important sites for identifying the essential features of the fission machinery. Since the complete loss of Drp1 is embryonic lethal, we propose that these mutations embody the most severe disruptions to protein function possible while still allowing for survival beyond early development. Individual mutations have been introduced into human Drp1 and purified by recombinant protein expression. Preliminary results indicate several mutant proteins remain capable of oligomerization and all mutants maintain GTPase activity, with some possessing a higher basal rate of GTP hydrolysis than WT. The deficiency of some mutants appears to be in lipid-binding, but other mutants maintain an enhanced rate of GTP hydrolysis on a lipid template, highlighting the functional heterogeneity among these mutations. Ultimately, these studies will provide insight into the structure-function relationship of Drp1 and identify the specific defects in Drp1 activity that give rise to human pathologies.

P1189

DRP-1 and Mitochondrial-associated Endoplasmic Reticulum Membranes are Important for Associated Fission Induced Exercise

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Diabetes has affected 29.1 million people in the US, and another 86 million have prediabetes. It has been shown that regular exercise (Ex.) has emerged as one of the best therapeutic strategies to prevent and treat Diabetes. Ex. has shown to increase levels of Dynamin-related protein 1 (Drp-1), increase
Mitochondria-associated Endoplasmic Reticulum membranes (MAMs) formation, increase mitochondrial biogenesis, and increase autophagy. DRP-1 is a cytosolic GTPase protein that plays a central role in mitochondrial fission during Ex. with the help of MAMs. However, it is unknown if MAMs can influence mitochondrial fission in the absence of DRP-1 after Ex. We hypothesized that mitochondrial fission can occur in the absence of DRP-1 in skeletal muscle after Ex. We used CRISPR technology to knock out DRP-1 in C2C12 myotubes and primary myotubes and then used the electrical stimulation (e-stim) paradigm to mimic Ex. L-lactate and glucose assay was employed to determine if e-stim was sufficient in inducing an Ex. response. To date, we were able to demonstrate that e-stim in C2C12 myotubes cells and primary myotubes exhibited a 2 ½ fold increase in L-lactate and manifested a 1/3 decrease in free glucose. In contrast, primary human myotubes did not show a decrease in glucose and exhibited a 2 ½-fold increase in L-lactate. Notably, we observed by western blot that there was an increase DRP-1 total levels, phosphorylation of AMPK alpha at threonine 172, phosphorylation of AMPK beta at serine 182, and phosphorylation of DRP-1 at serine 616 were increased after electrical stimulation in C2C12 and primary myotubes. Western blot analysis also demonstrated that ER marker, calreticulin was increased and demonstrated that MAM proteins, MFN-2, and GRP75 were increased in C2C12, primary, and human myotubes. TEM analysis demonstrated that the MAM distance decreased after Ex. Next, we ablated DRP-1 in myotubes for 7 days. TEM analysis demonstrated that loss of DRP-1 resulted in hyperbranched mitochondria, increased lipid droplet accumulation, altered cristae morphology, ER surface area expansion, and widened MAM distance. C2C12 and Primary Drp1 KO myotubes were subjected to acute and chronic e-stim paradigms and demonstrated a 3 and 3 ½ fold decrease in available free glucose, respectively. To determine if actin polymerization in the absence of Drp1 can promote fission, we used the actin polymerization Biochem Kit to access this question and the result indicated a decrease at basal condition (baseline of actin polymerization) for Drp1 KO primary myotubes, and a decrease in G-actin polymerization for Drp1 KO primary myotubes. In summary, we were able to validate the efficacy of our e-stim protocol and CRISPR technology. Our results also may suggest that loss of DRP-1 in myotubes require more glucose to Ex.

P1190

A Charcot-Marie-Tooth Type 2A Mitofusin Variant Alters Mitochondrial Fusion and Cellular Distribution
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The ability of mitochondria to move, fuse and divide is necessary for mitochondrial function and cell health. The molecular mechanisms of mitochondrial fusion are not well understood. Mitochondrial fusion is mediated by two conserved mitofusin (Mfn) paralogs, Mfn1 and Mfn2. The mitofusins are members of a family of large GTPases that remodel membranes. In the current model for mitochondrial fusion, mitofusin forms an intermitochondrial tether preceding membrane fusion. Mutations in MFN2 are associated with the peripheral neuropathy, Charcot-Marie-Tooth Type 2A (CMT2A). We have characterized a disease-associated variant of Mfn2 and the paralogous amino acid substitution in Mfn1 (Mfn2-S350P and Mfn1-S329P). Expression of either variant in mouse embryonic fibroblasts led to a dramatic coalescence of mitochondria in the perinuclear space. Although this position is within the catalytic domain, we have determined that the enzymatic activity of the Mfn1 variant is similar to wild type. Although the substitution does not alter intrinsic enzymatic activity, GTPase function is necessary for the redistribution of mitochondria that results from expression of either Mfn1-S329P or Mfn2-S350P.
To investigate whether mitochondria within the cluster are connected, we targeted photoactivatable GFP to the mitochondrial matrix. Following activation of a small region, we observed that GFP did not readily diffuse to the rest of the mitochondrial cluster expressing Mfn1-S329P, indicating that mitochondria are not connected. To study the dynamics of mitochondrial movement to the perinuclear space, we developed an inducible expression system in which either wild type mitofusin or the proline variant is expressed upon addition of tetracycline in HEK293 cells. We observed rapid redistribution of mitochondria following expression of the CMT2A-variant, but not wild type Mfn1 or Mfn2. Therefore, the change in mitochondrial distribution is rapid, suggesting an active process. Once formed, the mitochondria within the cluster do not undergo fusion nor readily move away from the perinuclear space. Together, our data suggest that these mitofusin variants form intermitochondrial tethers that cannot progress to fusion and are not readily reversible. From this, we suggest that mitofusin must be activated by GTP binding and conformational changes in order to form an intermitochondrial tether.

P1191

The evolution of the mitochondrial inner membrane remodeling dynamin-related GTPases Mgm1 and Opa1

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Mgm1 and Opa1 are known as central players in both mitochondrial inner membrane (IM) fusion and cristae remodeling in fungi and animals, respectively. Their uncanny similarity in domain architecture and function ostensibly suggests they are directly related orthologs, although this view has been challenged. To shed light on their relationship, we carried out bioinformatic and phylogenetic analyses benefiting from the expansion of sequence data from diverse protists. Our results suggest that Mgm1 and Opa1 form two different clades of the dynamin family not directly related to each other. Moreover, the C-terminal region of Opa1 has diverged considerably from Mgm1 and other dynamin-related proteins. Opa1 is not restricted to Holozoa (i.e. animals and their closest unicellular relatives) as originally supposed, since we found it also in representatives of CRuMs, a more distantly related supergroup containing free-living protists. This suggests that Opa1 evolved quite early in eukaryotes and was lost independently in several lineages. Contrarily, Mgm1 remains restricted to Fungi and their closest relatives. The possibility that Mgm1 is a transmogrified version of Opa1 is unlikely, as this would necessitate a reversion of its atypical C-terminal region into the more conventional form present in Mgm1. When looking for Mgm1 and Opa1 homologs, we encountered a previously unnoticed dynamin family superclass we dub “MidX”. It exhibits a very peculiar distribution, being found in a couple of distantly related lineages and surprisingly also in several giant viruses. The MidX domain architecture is similar to that of Mgm1, except for the apparent absence of a transmembrane domain. To address the possibility that MidX may be imported into the mitochondrial matrix we demonstrate that MidX from a giant soil virus is targeted to the mitochondrion when exogenously expressed in the discoban Trypanosoma brucei. Indeed, viral infections of various hosts are associated with changes to mitochondrial morphology, perhaps representing a part of an elusive viral reproductive strategy. To this end, MidX may remodel the IM from the matrix side.
P1192

Regulation of cancer stem cell metabolism and mitochondrial dynamics by extra cellular matrix

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Cancer stem cells (CSCs) are a subpopulation of cells within a tumour that have stem cell-like properties with tumorigenic capacity, making them a driver of metastasis and therapeutic resistance. Like other stem cells, CSCs are suggested to have a distinct metabolic profile, with recent reports indicating that CSCs can switch their metabolic phenotypes between glycolytic and OXPHOS-dependent pathways according to their microenvironment. The key metabolic hub, mitochondria are also altered in CSC, although different groups reported conflicting results in its structure and function. As these unrecognized pathways regulating mitochondria in CSC promoting growth and survival is unknown, we set to identify these factors. Here, we show that CSC attachment to the extra cellular matrix (ECM) drives changes in mitochondrial structure and impact their function. To study the role of the ECM in the regulation of CSC, we generated CSCs from different breast cancer cell lines. We then attached the CSC to different ECM substrates and measured mitochondrial structure and function. Specifically, CSCs generated from breast cancer cell lines in suspension showed fragmented mitochondria, but rapidly elongated their mitochondrial network upon attachment. This was associated with the oligomerization of mitochondrial inner membrane dynamin OPA1, which regulates mitochondrial fusion and cristae maintenance. Importantly, these changes did not affect cell viability, but regulated mitochondrial metabolism and function. Altogether, our results indicate that CSC mitochondrial structure and function is regulated by ECM attachment. Different microenvironment (for example cells within a solid tumour vs cells that have escaped the tumour microenvironment) could show distinct metabolic profiles independently of their genetic makeup.

P1193

All-trans retinoic acid has positive effect on mitochondrial dynamics and survival of cardiomyoblasts after laser-induced oxidative stress

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Introduction: The prevention of oxidative stress is beneficial during therapy of severe pathologies, including ischemia/reperfusion (I/R) period. The all-trans retinoic acid (ATRA) induces cardiac development and differentiation as well as the apoptosis reduction under I/R conditions. The mitochondria are sensitive to oxidative stress. The objective of this study was to determine the effect of ATRA on the ability of mitochondria to maintain its membrane potential (ΔΨm) and on mitochondrial dynamics after severe oxidative stress induced by laser irradiation. Methods: H9C2 cardiomyoblasts were treated with ATRA (10nM or 1µM) for 2-4 days. The measurement of ΔΨm by fluorescent TMRM
was used also for image analysis of morphology and motility of mitochondrial networks. To assess the mitochondrial dynamics, the protein levels of mitofusin 1 and 2 were measured by western blot. The simultaneous fluorescence visualization of ROS by H$_2$DCFDA and $\Delta\Psi_m$ allowed us to detect the reaction of H9C2 to the oxidation stress after photodamage. The mRNA level of retinoic acid receptors (RARα, RARβ, RXRα, RXRγ) was determined by real-time PCR. The preliminary protein screening was performed by liquid chromatography and mass spectrometry-based proteomics. **Results:** The low-dose ATRA (10nM) stimulated cells had lower basal level of ROS than untreated cells. When exposed to laser irradiation, these cells could maintain $\Delta\Psi_m$ with better survival rate. The increase of mitochondrial motility was significantly detected in low-dose ATRA stimulated cells. Significant increase of mitochondrial junctions was connected with increased expression of Mitofusin1. These changes were not present when the cells were stimulated with higher dose of ATRA (1µM). The low-dose ATRA (10nM) stimulated the increasing expression of retinoic acid receptors (2- to 4-fold) whereas the higher dose (1µM) did not. By proteomic screening, we revealed increase of protein expression with antioxidant activity (peroxiredoxins), which could contribute to protective effect of ATRA in cardiomyoblasts. While the 10nM ATRA had overall positive effect on the cardiomyoblasts under severe oxidative stress, 1µM ATRA caused uncertain and not significant changes in all studied parameters. This might be related to the ligand-dependent degradation bound to nuclear retinoic acid receptors. **Conclusions:** The protective effect of ATRA on cardiomyoblasts exposed to oxidative stress is connected with induction of changes in mitochondrial morphology and dynamics, lowering of basal ROS level, stabilization of $\Delta\Psi_m$ and stimulation of the expression of proteins having antioxidant activity. **Funding:** The research was funded with VEGA 2/0090/18 (KM), VEGA 2/0121/18 (MF), APVV 15-0372 (JB, DM) and APVV 15-0119 (MF).

**P1194**

**Macrophage activation alters mitochondrial morphology and promotes mitochondrial transfer to cancer cells**

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Macrophages contribute to a diverse set of functions including tissue homeostasis, immune responses, and tumor clearance. In some circumstances, macrophages also contribute to tumor progression, however this is not well understood. It has been shown that cells can directly transfer mitochondria to increase survival of recipient cells, however macrophage mitochondrial transfer has not been fully investigated. By co-culturing human macrophages and human cancer cells, we observed that macrophages transfer mitochondria to cancer cells, resulting in enhanced tumor cell proliferation. In a process called macrophage activation, environmental cues dictate macrophages to undergo transcriptional and phenotypic changes, eliciting their diverse functionality. Activated macrophages are commonly classified as M1 or M2 macrophages, which promote inflammatory or pro-tumorigenic processes, respectively. Aiming to better understand how M2 macrophages may contribute to tumor progression, we tested how macrophage activation influenced macrophage mitochondrial transfer and found that M2 macrophages exhibit enhanced mitochondrial transfer rates to tumor cells when compared to M1 macrophages. To investigate how macrophage activation could promote mitochondrial transfer, we sought to determine whether macrophage activation influenced mitochondrial dynamics and morphology. We hypothesized that macrophages with fragmented mitochondrial networks would be more likely to transfer mitochondria than macrophages with highly-fused mitochondrial networks.
Through quantitative analyses we determined that M2 macrophages contain a 4-fold increase in the number of small mitochondrial fragments compared to M1 macrophages. Aiming to directly determine how mitochondrial morphology influenced mitochondrial transfer, we knocked down a key driver of mitochondrial fission, Drp1, in macrophages. This genetic perturbation resulted in highly fused mitochondrial morphology, 1.9 fold decrease in mitochondrial fragmentation and did not influence macrophage activation status. By co-culturing Drp1-knock down macrophages with tumor cells, we observed that preventing mitochondrial fragmentation in macrophages reduced mitochondrial transfer rates to cancer cells. These studies suggest that macrophage activation contributes to changes in mitochondrial morphology, and that these changes influence mitochondrial transfer to tumor cells. Investigating how macrophage activation influences macrophage-to-tumor mitochondrial transfer will help further elucidate the diverse role of macrophage function and help characterize an unappreciated mechanism of M2 macrophage associated tumor progression.

**Modifications in Intracellular Signaling**

**P1195**

**The membrane mucin MUC17 and its role in intestinal homeostasis and inflammation**

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Our intestines constitute a semi-permeable barrier which allows nutrients absorption whilst pathogens are kept at a distance. This interface is made possible by heavily glycosylated proteins called mucins that line our epithelium by either being anchored to the membrane of enterocytes or by being secreted out into the intestinal lumen by goblet cells. The mucin MUC17 is one of the major membrane mucins expressed by enterocytes. Our group has recently shown that MUC17 is specifically upregulated during weaning and forms the glycocalyx covering the brush border of enterocytes. The glycocalyx functions as a barrier against exogenous factors such as bacteria. We have previously shown that correct localization of MUC17 to the brush border of enterocytes requires an interaction with the scaffolding protein PDZK1. Moreover, MUC17 harbors two C-terminal phosphorylation sites, one of which lies within the PDZK1-binding sequence. We hypothesize that, apart from forming a barrier towards the intestinal lumen, membrane mucin MUC17 interacts with several intracellular proteins that coordinate MUC17 trafficking, formation of the glycocalyx and relay signals from the luminal environment to the host enterocytes. We aim at identifying the phosphorylation-dependent interactome of MUC17 by combining a strategy of SILAC, reversible cross-link-immunoprecipitations coupled to mass spectrometry. We will use confocal microscopy and electron microscopy to study effects of MUC17 phosphorylation on MUC17 localization in relation to the formation of the glycocalyx and microvillar clusters during cellular differentiation. Using microscale thermophoresis and isothermal calorimetry, we will determine the binding parameters of MUC17 interacting with PDZK1 and assess how this interaction is affected by MUC17 phosphorylation within the PDZ-binding motif.
**Pyrophosphorylation-dependent disruption of RAD51-BRCA2 interaction promotes DNA damage repair**

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Homologous recombination (HR) mediated repair is a high fidelity mechanism by which DNA lesions are repaired during the S/G2 phase of the cell cycle. RAD51 plays a crucial role in DNA strand exchange during HR. However, little is known about the processes that facilitate the removal of RAD51 from the site of DNA repair, and enable the cell to re-enter the cell cycle. Inositol pyrophosphates are energy-rich small molecules found in all eukaryotes. The inositol pyrophosphate 5-IP7 (5-diphosphoinositol pentakisphosphate) is synthesized from IP6 (inositol hexakisphosphate) by IP6 kinases (IP6Ks), of which there are three paralogs in mammals - IP6K1/2/3. 5-IP7 modulates several cellular functions by triggering an enigmatic posttranslational modification called serine pyrophosphorylation. During this process, 5-IP7 transfers its β-phosphate to a pre-phosphorylated serine residue lying within an intrinsically disordered region of the target protein. We have earlier shown that 5-IP7 synthesized by IP6K1 is essential for DNA repair in mouse embryonic fibroblasts treated with hydroxyurea or neocarzinostatin. We now show that U-2 OS cells depleted for IP6K1 exhibit persistence of mitomycin C induced DNA damage foci marked by γH2AX and RAD51. Upon removal of the genotoxic stressor, cells with reduced IP6K1 showed a slower re-entry into the cell cycle compared with control cells. Surprisingly, knocking down IP6K1 did not reduce the efficiency of HR in a reporter assay, suggesting that the defect lies in the removal of repair proteins and not in the repair of DNA damage per se. Subsequent to HR-mediated repair, disruption of the interaction between RAD51 and the C-terminal domain (CTD) of BRCA2 helps to dislodge RAD51 from the repair site. We observed that increased expression of IP6K1 attenuates the interaction between RAD51 and the BRCA2 CTD, suggesting that IP6K1 promotes the post-repair removal of RAD51. However, a catalytically inactive form of IP6K1 does not influence the RAD51-BRCA2 CTD interaction, indicating that 5-IP7 is required for this effect. We show that IP6K1 interacts with both RAD51 and BRCA2 CTD, and that RAD51 can undergo serine pyrophosphorylation by 5-IP7. We propose a mechanism by which IP6K1 is recruited to the site of HR to produce 5-IP7, which in turn promotes the completion of DNA repair and removal of RAD51 via protein pyrophosphorylation.

**Modulation of Sts5 Impacts Heat Tolerance and Stress Granule Formation in Schizosaccharomyces pombe**

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Using the model organism *Schizosaccharomyces pombe*, we recently reported that the NDR/LATS kinase Orb6 impacts chronological lifespan of the cell by regulating the RNA binding protein Sts5. Under nutrient replete conditions, Orb6 phosphorylates Sts5, causing sequestration by the 14-3-3 protein Rad24. This allows for transcription of bound mRNA, and results in increased polarized growth and decreased chronological lifespan. Conversely, under nutrient poor conditions Sts5 is not phosphorylated and it coalesces into ribonucleoprotein (RNP) puncta, which results in decreased polarized growth and
increased chronological lifespan, as Sts5 bound mRNAs are translationally repressed. We previously demonstrated that Sts5 puncta co-localize with P-Bodies, a type of RNP granule which aids in mRNA degradation. Here, we found that Sts5 puncta also colocalize with another type of RNP granule known as stress granules (SG) in response to a broad range of stressors including heat, oxidative, and osmotic stress. Thus, we further investigated the role of Sts5 in the formation of SG and stress response. We found that loss of Sts5 correlates with decreased SG granule formation and also decreased survival following heat stress. Conversely, hyperactive Sts5 confers increased survival following heat stress. These data indicate that Sts5, in addition to its role in the nutrient dependent regulation of polarized growth and chronological lifespan, also impacts diverse stress responses. In particular, it is important for resistance to heat stress, and may aid in the seeding of stress granules under inducing conditions. In mammals, SG dysfunction can lead to neurological disease, cancer, and increased viral susceptibility. Therefore, further evaluation of Sts5 may yield important discoveries with implications for human disease.

P1198

Fast and fine-tuned: DEPTOR tyrosine phosphorylation offers new insight on mTOR activity

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Metabolism dysfunction is considered a major initiator of tumorigenesis. The serine/threonine kinase mTOR (mechanistic Target Of Rapamycin) is a central regulator of cellular metabolism and homeostasis. Constitutive activation of the mTOR pathways, resulting from upstream regulatory dysfunction, is now considered as a potent inducer of tumor growth and cancer cell survival. Indeed, mTOR regulates the modulation of cellular process such as, autophagy, macromolecule biosynthesis, cell mobility, cell survival, and cytoskeletal rearrangement. Due to its biological versatility, it is not surprising to observed altered mTOR activation in many cellular dysfunction and diseases. In many cancers, mTOR is constitutively activated due to a mutation in its canonical pathway (PI3K/PTEN/AKT). This involves cells to lose their homeostasis and gain cancer-like features required for their transformation. Nevertheless, some pathologies such as low-grade gliomas (LGG) shows a phenotypic constitutive mTOR activation without harboring mutations within this canonical pathway. Hence, non-canonical activation of mTOR pathways could enhance Low-grade gliomas transition into secondary glioblastomas (grade IV), which inevitably lead to increased lethality. Using mass spectrometry analysis on DEPTOR, an endogenous regulator of mTOR, we identified a novel phosphorylation event targeting a single tyrosine (Tyr289) that rapidly affects DEPTOR inhibitory function on both mTORC1&2. DEPTOR phosphorylation on tyrosine 289 promotes its dissociation from mTOR, thus allowing a rapid and sustained activation of both mTORC1&2. Previous work showed that DEPTOR post-translational modifications enhanced its proteasomal-induced degradation, thus the release of its inhibitory function. Conversely, our preset work described a novel molecular switch that temporarily inactivate the repressive functions of DEPTOR, while preventing its degradation, thus allowing a rapid inhibitory fuctions of DEPTOR on mTOR complexes. Hence, we pursue our investigation on the effect of this post-translational modification on DEPTOR role on mTOR activation in early phases of cancers progression, such as observed in LGG progression.Taken together, our findings identified a novel molecular mechanism involved in modulating mTOR activity, in a PTEN/PI3K/AKT-independent manner, as observed in a subset of cancer.
**Functional Impact of O-GlcNAc on Kelch-Like Family Proteins and Proteostasis**


Nutrient sensing is a cellular mechanism in response to environmental signals to maintain homeostasis. Directly connected with glucose metabolism through the hexosamine biosynthetic pathway, O-linked β-N-acetylgalactosamine (O-GlcNAc) is a monosaccharide post-translational modification on cytoplasmic, mitochondrial, and nuclear proteins. Previous work by our lab and the Chi Lab demonstrated the connection between nutrient sensing and proteostasis through O-GlcNAc regulation of the Kelch-Like (KLHL) family proteins. KLHL proteins are adaptors for E3 ubiquitin ligase complexes, mostly facilitating substrate ubiquitination and degradation. Our labs first identified O-GlcNAc regulation of Keap1 (KLHL19), which targets Nrf2, a transcription factor that activates oxidative stress responses, for proteasomal degradation. An unglycosylatable S104A mutation impairs the ability of Keap1 to target Nrf2 for degradation, indicating the functional importance of O-GlcNAc in Keap1 regulation. Since KLHL proteins are structurally and functionally conserved, we next studied the potential O-GlcNAc regulation of another KLHL family member, gigaxonin (KLHL16), which is implicated in a rare, autosomal recessive disease, gigant axonal neuropathy (GAN). Mutations in gigaxonin cause GAN, which is characterized by defects in targeting intermediate filament proteins for proteasome-mediated degradation. As a result, neurofilament proteins aggregate in axons, leading to peripheral and central nervous system dysregulation. Our studies on gigaxonin O-GlcNAcylation demonstrate that O-GlcNAc regulates the function of gigaxonin in targeting intermediate filament proteins for degradation, specifically at sites S272 and T277. O-GlcNAc regulation on gigaxonin is also nutrient-sensitive, as glycosylation decreases upon glucose, glutamine, and serum starvation. As a further test of our hypothesis that O-GlcNAcylation is a general regulatory mode for KLHL proteins, we present new data that several additional KLHL proteins may be O-GlcNAc-modified. Future studies will investigate the potential functional roles of O-GlcNAcylation on these proteins.

**Fringe mediated glycosylation is involved in Jagged1-dependent accumulation of CADASIL mutant NOTCH3**

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CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leuкоencephalopathy) is a typical form of heritable small vessel disease and caused by the mutation in **NOTCH3**, which is a gene coding transmembrane receptor for cell-cell signaling. The accumulation of mutant **NOTCH3** is believed to have a vital role in the pathogenesis of CADASIL. It is known that **NOTCH3** is degraded by a ligand, JAGGED1 (JAG1). Fringe is a glycosyltransferase for Notch and has crucial roles in the regulation of Notch signal activity. A previous study showed that Fringe mediated glycosylation was inhibited in some **NOTCH3** mutants. However, it is unknown whether Fringe affects the accumulation of the **NOTCH3** mutants. Therefore, we evaluated the effects of Fringe on the JAG1 dependent metabolism of **NOTCH3**. We established the **NOTCH3** (WT and C185R, a CADASIL mutant) and Fringe expressing cells,
and detected the increasing molecular mass of NOTCH3 by Fringe expression in WT and C185R cells by Western Blotting. We found that Fringe inhibited NOTCH3 signal activity by JAG1 both in WT and C185R cells. In addition, WT NOTCH3 was degraded by JAG1 with or without Fringe expression, while C185R NOTCH3 was degraded less with Fringe expression. These data suggest that Fringe may have a key role in the accumulation of mutant NOTCH3. Since NOTCH3 undergoes endocytosis into JAG1 expressing cells, the glycosylation of C185R NOTCH3 but not that of WT NOTCH3 may lead to the suppression of the NOTCH3 degradation in JAG1 expressing cells.

P1201

The role of cholesterol depletion in p75NTR mediated apoptosis
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Neuron death is an evolutionarily conserved process required for proper nervous system development. Survival and death decisions in the peripheral nervous system are mediated by the neurotrophin (NT) family, which includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). For each neurotrophin, there exists a specific Trk family receptor tyrosine kinase to mediate survival. For example, target organ derived NGF binds to TrkA in distal axons to mediate survival of sympathetic and nociceptive sensory neurons. Additionally, these neurotrophins, along with their pro-forms, can bind to the pan neurotrophin receptor p75NTR to mediate neuronal apoptosis. The balance between Trk receptors and p75NTR often determines whether a developing neuron will live or die. Here, we investigate the molecular mechanism of p75NTR mediated apoptotic signaling in sympathetic neurons. We present data showing that post-translational modification of p75NTR at cysteine residue (C279) by the addition of a palmitoyl group is necessary for apoptotic signaling. We generated a palmitoylation resistant mutant construct (C279A), to further investigate the role of p75NTR palmitoylation in apoptotic signaling. Palmitoylation of proteins mediates membrane attachment and can play an important role in regulating their signaling abilities, possibly via spatial segregation of signaling proteins into cholesterol-rich membrane domains. These domains are known as lipid rafts. To understand the mechanism involved in p75NTR mediated neuronal apoptosis, we will present data on whether cholesterol depletion affects p75NTR mediated neuronal apoptosis by regulating its localization to lipid rafts.

P1202

N-terminal regulation of potential therapeutic targets for NRas-driven cancers: the ABHD17 proteins
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NRas is a small GTPase that activates important signaling pathways at the plasma membrane and is commonly mutated in cancers. Cycling of NRas between the Golgi and the plasma membrane is required for oncogenesis and is enabled by the addition of a 16-carbon fatty acid palmitate, through a reversible bond. A family of proteins called the Alpha/Beta Hydrolase Domain-Containing (ABHD) 17 proteins cleave palmitate from NRas, which removes the protein from the plasma membrane, resetting the cycle. No therapeutics currently exist for NRas-driven cancers, but the ABHD17 proteins provide a potential druggable target. The ABHD17 proteins are largely uncharacterized, but they are known to have a
conserved, palmitoylated N-terminus that is necessary for plasma membrane localization and activity on NRas. Here, I show how the N-terminal cysteine residues of ABHD17A affect the localization, activity, and binding pocket accessibility of the enzyme. By appending a plasma membrane localization motif to the N-terminus of a palmitoyl-deficient ABHD17A, I show the necessity of membrane association for activity and binding pocket accessibility of the ABHD17 proteins. Understanding the importance of the N-terminus in regulating the activity of the ABHD17 proteins will aid in the creation of targeted therapeutics for oncogenic NRas.

P1203

**Pdz-rhogef is a novel ubch10-interacting protein that is targeted for degradation in mitosis by the anaphase promoting complex/cyclosome**

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UbcH10 is an E2-conjugating enzyme for the Anaphase-Promoting Complex/Cyclosome (APC/C) E3-ubiquitin ligase, which aids the APC/C-dependent ubiquitylation and proteasomal-mediated degradation of key cellular proteins during mitosis and the G1 phase of the cell cycle. UbcH10 is a proto-oncogene that is overexpressed in many human cancers; transgenic mice that overexpress UbcH10 are prone to APC/C over-activity, aneuploidy and tumour formation. We have used immunoprecipitation coupled to mass spectrometry to characterize the UbcH10 interactome and have identified a number of novel UbcH10-interacting proteins. Using this approach we have determined that PDZ-RhoGEF is a UbcH10-interacting protein that is targeted for degradation in early mitosis in a SAC-insensitive manner. In this regard we have established that UbcH10 overexpression, and the Cdk1-targeted phosphorylation of PDZ-RhoGEF promotes its degradation. The results of these studies will be presented and the implications of these findings discussed.

P1204

**Cell systems biology of proteins associated with AGC kinase Sch9**

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Sch9 appears to be the *Saccharomyces cerevisiae* homolog of protein kinase B and S6 kinase and is involved in the control of numerous nutrient-sensitive processes, including regulation of cell size, cell cycle progression, and stress resistance. Sch9 has also been implicated in the regulation of replicative and chronological life span. The availability of data from global studies of protein-protein interactions now makes it possible to predict and validate functional connections between Sch9, its putative substrates, and other proteins. Sch9 appears to be involved in control of biosynthetic and catabolic pathways. Thus, the analysis of Sch9-associated proteins indicates that this kinase may be involved in regulation of protein synthesis. Sch9 forms a complex with, and, presumably, phosphorylates Arc1, Pab1 and prion-like protein Sup35. Arc1 is a component of tRNA synthetase complex. Pab1 functions in association between the 5’ cap and 3’ mRNA poly(A) tail, and Sup35 functions in the termination of translation. Thus, Sch9 may be part of the mechanism that relays availability of nutrients to utilization of glucose and to the rates of protein synthesis. One of the interesting outcomes of the proteome-wide
analysis of protein-protein interactions in yeast is the finding that Sch9 associates with Shp1, Cdc48, and Ufd1, which form a complex responsible for the recognition and targeting of ubiquitinated proteins to the proteasome for degradation. What is the result of the interaction of Sch9 with this complex of the protein-degradation machinery? One possibility is that Sch9 itself is ubiquitinated and targeted for degradation by Shp1-Cdc48-Ufd1. An alternative scenario is that Sch9 phosphorylates some of the components of this complex to regulate its activity. It is unknown and remains to be elucidated, whether mammalian homologues of Sch9 are also associated with the proteins involved in translation/protein synthesis and proteasomal degradation. In my future work, I am interested to study the homologues and functional counterparts of this protein kinase in human genome and to identify the functional DNA sequence variants of these proteins and their roles in normal cell biology, cancer and aging-associated diseases.

**Motile Cilia Dynamics**

P1205

**Putting cilia in motion: Structure of the radial spoke head**

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Motile cilia power cell locomotion and drive extracellular fluid flow in mammalian tissues by propagating bending waves from their base to tip. The coordinated bending of cilia requires mechanoregulation by the radial spokes (RSs), a 23-protein complex that extends between the microtubule outer doublet and the microtubule central pair (CP). Despite their importance for cilia motility across eukaryotes, the molecular function of the RSs is unknown. We reconstituted the RS head that abuts the CP and solved its structure using single-particle cryo-electron microscopy to 3.1 Å resolution. The structure, which includes models for nine proteins built de novo, reveals that the RS head has a remarkably flat, negatively-charged surface that faces the CP and is supported by a rigid core of tightly intertwined proteins. Partially reversing the negative charge on the RS surface impaired cilia motility in the unicellular organism *Chlamydomonas reinhardtii*. Human ciliopathy-based point-mutations in the RS head compromised stability of the recombinant RS complex, providing a molecular basis for understanding disease. This structural work reveals that the RS head is optimally designed to physically collide with and repel the CP during a mechanically-induced bend, a process that may underlie the regulation of rhythmic ciliary beating.

P1206

**Helical navigation and the origins of bilateral symmetry-breaking in Chlamydomonas**

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Organisms perceive the world as three-dimensional. Unicellular self-propelled microalgae often adopt chiral or helical trajectories as they navigate through bulk fluid. The ubiquity of helical swimming suggests that periodic environmental scanning enhances the fidelity of cellular responses to vectorial stimuli or cues, e.g. light, or the presence of prey organisms. The model biflagellate alga *Chlamydomonas reinhardtii* rotates at a constant 1-2 Hz about its body axis as it swims, but to date the
mechanism for this has not been confirmed. We prove for the first time that this rolling motion derives from a consistent, non-planar flagellar beat pattern. We demonstrate beat non-planarity by high-speed imaging and micromanipulation of live cells from above (direct anterior view). In order to relate the observed flagellar beat patterns directly to the free-swimming dynamics, we constructed a fully 3D theoretical model of the biflagellate. Incorporating geometrical parameters inferred from the experimental data, our model is able to reproduce both the sense and magnitude of the axial rotation observed in real cells. In particular, we deduce that helical swimming arises from unequal flagellar beating. Moreover we find that cells are able to reorient towards or away from directional stimuli for positive or negative phototaxis by actively modulating this bilateral asymmetry. We conjecture that molecular and physiological differences between the cis and trans flagella (which are known to differ in terms of basal body age) underlie intracellular control over biflagellar dominance, which is critical for any form of steering response or taxis.

P1207

**How Cells Search**

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Efficiently searching space is a key requirement for microorganisms to gather information, find food, mates and make behavioral decisions. The ciliate *Lacrymaria olor* presents a striking example of such a "search" behavior wherein a single-cell rapidly extends a neck-like protrusion to several times its body size to dynamically and efficiently search its surroundings. This behavior is all the more remarkable given the physical environment of the cell which is highly dissipative, making such dynamic movements challenging. How cells encode such a complex physical behavior by regulating their underlying cytoskeletal, and ciliary machinery over multiple length and time-scales is currently not understood. Here we present a framework for how cells search by combining experiments using the ciliate *Lacrymaria olor* and a biophysical model based on soft, active filament hydrodynamics. Using this model, as well as computational, high-throughput microscopy of cell postures, we report a novel scrambling mechanism of the neck that emerges due to an interaction between motile cilia and the emergent cell mechanics due to the underlying cytoskeleton. We explore the phase space spanning the cell's emergent mechanical properties and ciliary activity, and identify regions where this search behavior is particularly efficient, thus building a framework for interpreting experimental measurements of cell postures. Overall, our combined approach points to a rich space of cell behaviors even beyond "searching" that can be programmed using this curious interplay of motility and morpho-dynamics.

P1208

**Tetrahymena thermophila** basal body connections promote ciliary synchrony

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Multi-ciliary arrays comprise of fields of cilia that beat in synchrony to promote efficient fluid flow and directed cellular motility. Ciliary synchrony or the coordinated beating between cilia is well established to rely on hydrodynamic coupling to synchronize the beating of neighboring cilia. However, hydrodynamic coupling alone is insufficient to generate ciliary synchrony in some organisms (Wan
In addition to hydrodynamic coupling, cilia are also intracellularly connected via underlying cortical architecture. Intracellular connections are required for coordinated ciliary mobility (Wan & Goldstein (2019)). How intracellular connections promote ciliary synchrony remains poorly understood. The well-established cortical architecture of *Tetrahymena thermophila* serves as an excellent system to elucidate the functional significance of intracellular connections in promoting ciliary synchrony. *Tetrahymena* ciliary arrays consist of longitudinally arranged ciliary rows. Each cilium is nucleated by a basal body (BB) that is anchored to the cell cortex. Proper BB positioning and polar orientation are promoted by BB connections. Each connection consists of an anteriorly oriented BB-associated striated fiber (SF) that interacts with the neighboring BB’s posteriorly oriented post-ciliary microtubule (pcMT) bundle. To investigate the functional role of BB connections in promoting ciliary synchrony, we utilized the SF mutant, *disA-1*, that exhibits BB disconnections, BB disorientation, and disrupted cell motility. As the cilia of *disA-1* cells remain motile, we hypothesized that compromised BB connections disrupt ciliary synchrony. To visualize and quantify ciliary dynamics, we established a novel method, Delivered Iron Particle Ubiquity Live Light- (DIPULL), to immobilize live *Tetrahymena* cells. Combining DIPULL with light microscopy, we quantitatively established the ciliary dynamics of wildtype *Tetrahymena* cells. Here we show that wildtype *Tetrahymena* cilia exhibit a 3-dimensional ciliary waveform. Cilia kinetics analysis revealed that the power stroke occurs approximately twice as fast as compared to the recovery stroke. In addition, *Tetrahymena* ciliary beat frequency is unique along the cell length whereby cilia in the anterior and medial regions beat faster than those in the posterior region. Conversely, *disA-1* mutants display aberrant ciliary waveforms. Moreover, they exhibit slower and more variable ciliary dynamics as compared to wildtype cells. Collectively, this suggests that proper BB connections are required to establish ciliary synchrony between neighboring cilia and thus enable efficient generation of fluid flow to perform cellular and extracellular motility.

**P1209**

**High hydrostatic pressure induces vigorous flagellar beating in *Chlamydomonas* non-motile mutants lacking the central apparatus**

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The beating of eukaryotic flagella (also called cilia) depends on the sliding movements between microtubules powered by dynein. In cilia/flagella of most organisms, microtubule sliding is regulated by the internal structure of cilia comprising the central pair of microtubules (CP) and radial spokes (RS). *Chlamydomonas* paralyzed-flagella (*pf*) mutants lacking CP or RS are non-motile under physiological conditions. Using high-pressure microscopy, here, we show that high hydrostatic pressure induces vigorous flagellar beating in *pf* mutants [1,2]. The beating pattern at 40 MPa was similar to that of wild type at atmospheric pressure. This is the first in vivo observation of beating in CP/RS-lacking cilia/flagella. Demembranated axonemes of these mutants also display beating upon addition of ATP at high pressure; therefore, pressure must induce beating by directly acting on the axoneme. Mutant flagella lacking outer arm dynein (OAD) together with the CP or RS do not display beating at high pressure, suggesting the involvement of OAD activity in pressure-induction of beating. This is similar to the previous results, at which the demembranated *pf* mutant axonemes could be activated in vitro in the presence of ATP plus salts or organic compounds [3]. In addition, at 80 MPa, flagella underwent an asymmetric-to-symmetric waveform conversion, similar to the one triggered by an increase in intra-
flagella Ca\(^{2+}\) concentration during cell’s response to strong light. Thus, high hydrolytic pressure seems to increase intra-flagella Ca\(^{2+}\) concentration, in addition to inducing flagellar beating in the pf mutants.


**P1210**

**Dancing basal bodies: basal bodies bend in response to ciliary force**

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Motile cilia beat asymmetrically to generate directed fluid flow and cell motility. Basal bodies (BBs) nucleate and anchor motile cilia, acting as the physical linkage to transmit forces from beating cilia to the cell for fluid mobility. BBs are composed of nine triplet microtubules arranged by linking proteins to form a nm-scale cylinder. BBs are 1/10th the length of cilia and lack ciliary dynein motors that generate the ciliary beat stroke. Additionally, in contrast to ciliary doublet microtubules, BBs are formed of triplet microtubules. Thus, relative to cilia, basal bodies have largely been considered to be rigid anchorage structures that are structurally unaffected by beating ciliary forces. However, specific BB mutants produce unstable BBs that exhibit ciliary force dependent BB structural defects. We asked whether BBs are normally dynamic structures that resist ciliary forces. Using electron microscopy imaging techniques (HPF-FS / EM tomography), we captured BBs and cilia during the ciliary beat stroke to visualize BBs relative to cilia position in their beat stroke. BBs undergo consistent physical deformation at defined triplet microtubules during the ciliary beat pattern. This suggests that indeed ciliary beating forces produce dynamic changes to BBs. In addition, BBs display movements relative to cilia position in the beat stroke. These data provide the first description of BB structural dynamics and BB movements relative to ciliary beating.

**P1211**

**Actin promotes ciliary motility in *Tetrahymena thermophila***

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Motile cilia beat in a coordinated fashion to effectively move fluids across cell surfaces. The role for the microtubule cytoskeleton and dynein is well characterized for ciliary beating, however two significant gaps in the field remain to be addressed: 1) the mechanics for how hundreds of cilia in multiciliary arrays beat in a coordinated manner and 2) the role of actin in ciliary function remains poorly defined for a single cilium and for ciliary arrays. One critical feature of coordinated ciliary motility within multiciliary arrays is the spatial organization of cilia. The position of cilia is precisely patterned at the cortex by basal bodies (BBs). In *Tetrahymena thermophila*, BBs anchor to the cell cortex through three BB-appendage structures that physically bridge neighboring BBs to each other and to the cell cortex. The coupling of BBs together and to the cortex creates a structured matrix to dissipate the forces generated by ciliary beating and to translate such forces to the cell. This is most evident by the finding that loss of cortical actin leads to beating cilia being ripped from the cell cortex in animal cells. However, the mechanism for
cortical actin anchoring BBs to the cell cortex in multiciliated cells has yet to be determined. Here, we begin to investigate the role for cortical and ciliary actin in ciliary function using *Tetrahymena* as a model organism. Interestingly, actin (Act1) has been identified in the basal body proteome and Act1-KO cells exhibit motility defects. To understand how actin may contribute to motility, we visualized F-actin using confocal microscopy. We find that F-actin is cortically enriched and localizes between BB-appendages to potentially facilitate cortical stabilization during ciliary beating. In addition to defining F-actin localization patterns, we begin to uncover the role of actin in *Tetrahymena* by assessing ciliary defects in Act1-KO cells. Taken together, our results support a requirement of actin in ciliary motility.

Neuronal Degeneration and Regeneration

P1212

The context of expanded GGCCTG repeats impacts the repeat toxicity in fly models of SCA36

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SCA36 (Spinocerebellar Ataxia 36) is a neurodegenerative disorder that is caused by a GGCCTG hexanucleotide repeat expansion in the first intron of *NOP56* gene. How such repeat expansion causes SCA36 is largely unknown. To examine the potential pathogenic mechanisms that lead to SCA36, we assembled 100 pure GGCCTG repeats from a short synthetic GGCCTG repeat sequence, and generated transgenic fly lines with the 100 pure repeats embedded with different sequence contexts and with different reporter tags to model human SCA36. Transgenic flies carrying the expanded repeats were crossed with specific drivers for tissue specific expression. When the pure repeats were expressed in the eyes, they caused rough eye phenotype in select lines, a characteristic phenomenon in fly models of neurodegenerative diseases. The severity of the rough eye phenotype were associated with shorter 3' expression tags and also seemed to be dependent on the expression level. Our results suggest that the expanded GGCCTG hexanucleotide repeats toxicity is dependent on the specific contexts, which can partially explain why *NOP56* to be the host gene of these expanded repeats for the disease.

P1213

Reducing heparan sulfate sulfation prolongs survival and reduces amyloid deposition in prion-infected mice

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Heparan sulfate proteoglycans (HSPGs) are major constituents of the brain extracellular matrix, neural glycocalyx and blood-brain barrier. HSPGs are associated with amyloid-β (Aβ) plaques and neurofibrillary tangles in Alzheimer’s disease, and with prion plaques in Creutzfeldt-Jakob disease. Genetic depletion of neuronal heparan sulfate (HS) reduced Aβ plaque deposition and accelerated Aβ clearance in APP mice. The interaction of HS with Aβ and PrP proteins is modulated by the length and the sulfation of the HS chains. We previously found that shortening HS chains prolonged survival and redistributed parenchymal prion plaques to blood vessels in prion-infected mice, which suggests that impairing the HS-prion interaction may also increase prion clearance into the CSF. Here we investigated how
decreasing HS sulfation impacts the replication of prions in vivo. *Ndst1*+/SYnCre+ mice, which express reduced neuronal HS sulfation due to the conditional deletion of the *Ndst1* gene in neurons, displayed 50% longer survival compared to *Ndst1*−/SYnCre- mice when infected with prions. *Ndst1*+/SYnCre+ brains showed 60% reduced prion levels and a redistribution of prion parenchymal plaques to vasculature and periventricular areas. Prion plaques in *Ndst1*+/SYnCre+ brains were also smaller and more fragmented, which was associated with an increased solubility of prion aggregates. However, decreasing HS sulfation did not seem to alter the conformation of the prion aggregates, as the biochemical properties of the prions in *Ndst1*+/SYnCre+ and *Ndst1*−/SYnCre- brains, including electrophoretic mobility, glycoprofile, and aggregate stability in guanidine chloride, were indistinguishable. Collectively, our results suggest that HSPGs trap prions in the brain extracellular matrix promoting their assembly into insoluble aggregates, potentially preventing their clearance into the CSF. Our study provides a new target for the rational design of neuroprotective therapies based on manipulating HS sulfation to disrupt the interaction of HS with misfolded prion protein.

P1214

**Fus overexpression is linked to altered histone post-translational modifications in an Amyotrophic Lateral Sclerosis yeast model.**

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Amyotrophic Lateral Sclerosis (ALS) is a debilitating and incurable disease. As our population ages, the incidence of ALS has increased and we still know little about hat causes it. Further complicating matters, the overwhelming majority of diagnoses are sporadic, meaning there is no family history of the disease. Recent evidence points to the contributions from epigenetic mechanisms to ALS disease etiology and progression. Although the majority of ALS cases do not involve a specific gene mutation, over 40 genes have been associated with the disease, including FUS. Epigenetics is broadly defined as any change in gene expression where the underlying DNA sequence is unaltered. Chromatin, a complex primarily involving DNA and histone proteins, controls access to the DNA. Histones post-translational modifications (PTMs) help regulate transcription by controlling access to transcriptional machinery. We have previously categorized the histone PTM landscape of a FUS overexpression yeast model of ALS, finding that there is significantly decreased levels of histone H3 phosphorylation at serine residue 10 (H3S10ph) and H3 acetylation at lysine residues 14 (H3K14ac) and 56 (H3K56ac). We now show that inhibiting protein phosphatase 1/2A (PP1/2A) with Okadaic Acid (OKA) and histone deacetylases (HDACs) with Trichostatin A (TSA), which are responsible for the removal of the histone PTMs H3S10ph and H3K14/56ac, respectively, reduce the toxicity associated with FUS overexpression in yeast. Our results suggest that epigenetic mechanisms play an important role in ALS and are an attractive new target in the development of novel therapeutics and treatments for this devastating disease.

P1215

**Impaired NHEJ repair in amyotrophic lateral sclerosis is associated with TDP-43 mutations**

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Pathological forms of TAR DNA-binding protein 43 (TDP-43) are present in motor neurons of almost all amyotrophic lateral sclerosis (ALS) patients, and mutations in TDP-43 are present in ALS. Loss and gain of TDP-43 functions are implicated in pathogenesis, but the mechanisms are unclear. While the RNA functions of TDP-43 have been widely investigated, its DNA binding roles remain unclear. In our recent study (Konopka et. al. Molecular Neurodegeneration, accepted 2020) we used NSC-34 motor neuron-like cells and primary cortical neurons expressing wildtype TDP-43 or TDP-43 ALS associated mutants (A315T, Q331K), in which DNA damage was induced by etoposide or H2O2 treatment. We investigated the consequences of depletion of TDP-43 on DNA repair using small interfering RNAs. Specific non homologous end joining (NHEJ) reporters (EJ5GFP and EJ2GFP) and cells lacking DNA-dependent serine/threonine protein kinase (DNA-PK) were used to investigate the role of TDP-43 in DNA repair. To investigate the recruitment of TDP-43 to sites of DNA damage we used single molecule super-resolution microscopy and a co-immunoprecipitation assay. We also investigated DNA damage in an ALS transgenic mouse model, in which TDP-43 accumulates pathologically in the cytoplasm. Finally, we examined fibroblasts derived from ALS patients bearing the TDP-43 M337V mutation for evidence of DNA damage. We demonstrate that wildtype TDP-43 is recruited to sites of DNA damage where it participates in classical NHEJ DNA repair. However, ALS-associated TDP-43 mutants lose this activity, which induces DNA damage. Furthermore, DNA damage is present in mice displaying TDP-43 pathology, implying an active role in neurodegeneration. Additionally, DNA damage triggers features typical of TDP-43 pathology; cytoplasmic mis-localisation and stress granule formation. Similarly, inhibition of NHEJ induces TDP-43 mis-localisation to the cytoplasm. This study identifies an important DNA-specific function of TDP-43 that is perturbed in ALS and highlights the emerging role of DNA damage in ALS development.

P1216

Genomic instability drives the neurodevelopmental and neuroinflammation defects due to RibonucleaseH2 loss in mice
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Aicardi-Goutières syndrome (AGS) is a monogenic type I interferonopathy that is characterized by severe brain dysfunction, cerebellar atrophy, upregulation of type I interferon signaling and neuroinflammation. Mutations in various genes that function in nucleic acid metabolism, including Rnaseh2 are linked to AGS. Ribonuclease H2 (RNaseH2) is a genome surveillance factor that protects DNA by removing RNA-DNA hybrids and ribonucleotides incorporated into the DNA during replication. The central nervous system is severely affected in AGS, although it is unclear why Rnaseh2 mutations results in neurological symptoms. Moreover, a key question is if persistent upregulation of type I interferon signaling or DNA damage drive the AGS pathology. Here we investigate how brain-specific deletion of RNaseH2 affects the nervous system in mice. Contrary to previous findings, we show that loss of RNaseH2 in the mouse brain profoundly affects early neurogenesis resulting in cerebellar
atrophy, activation of interferon-responsive genes and neuroinflammation. The Neuroinflammation is not coincident with ribonucleotide mediated DNA damage, but occurs after defective neurodevelopment. Furthermore, the cerebellar atrophy due to RNaseH2b deletion is rescued by p53 inactivation suggesting that DNA damage signaling, rather than neuroinflammation, is responsible for neuronal loss. Importantly, p53 activation in RNaseH2-deleted brains is not solely dependent upon ATM kinase activity as deletion of Atm did not rescue the cerebellar phenotype. In fact, coincident deletion of Atm and Rnaseh2 resulted in a worsened cerebellar phenotype dependent upon activation of non-homologous end-joining (NHEJ) leading to mice developing ataxia. The loss of ATM also exacerbates neuroinflammation. Thus, ATM suppresses the genomic damage and neuroinflammation after RNaseH2 inactivation by inhibiting toxic NHEJ. Collectively, our data suggest that it is DNA damage-dependent signaling rather than type I interferon upregulation that underlies the neurodegeneration in AGS, thereby providing critical insight into understanding how RNaseH2 and ATM prevent neurologic disease.

Neuronal Morphogenesis

P1217

14-3-3 shuttles ADNP to the cytoplasm to promote neuronal morphogenesis and functional cortical connectivity.
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Defective neuritogenesis is a contributing pathogenic mechanism behind a variety of neurodevelopmental disorders. Mutations in Activity-dependent neuroprotective protein (ADNP) are among the most frequent underlying genetic autism spectrum disorder (ASD). Frameshift and truncating mutations in ADNP result in a variety of complex neurodevelopmental symptoms. If defective neuritogenesis underlies the pathology of ADNP syndrome has yet to be explored. The objective of this study was to perform a detailed analysis for how loss of Adnp influences neuronal shape in the developing mouse brain. We further aimed to investigate whether pathological changes to neuronal shape had large scale consequences for brain function. We found that knockdown of Adnp using in utero electroporation of mouse layer 2/3 pyramidal neurons in the somatosensory cortex leads to neurite formation defects beginning at P0. We used ex vivo live imaging on brain P0 slices to observe defective neurite formation in real-time. In Adnp deficient neurons, we found serious flaws in cellular dynamics including failure of neurite retraction, slow growth speed, increased neurite stabilization, and intracellular swellings on growing neurites. These defects are sustained and worsened throughout development, at least through P15. The most notable defects at P15 include increased basal dendrite number, axon length, and interhemispheric axon innervation. Proper neuronal architecture is essential to neural network formation. Even slight changes to axon or dendrite morphology can lead to large scale changes in brain connectivity and function. To assess changes to neuronal function, we performed ex vivo calcium imaging which revealed that Adnp deficient neurons had greater spontaneous calcium influx. To further probe changes to neuronal activity, we analyzed whether excitatory connectivity was altered in Adnp deficient neurons. We utilized GPI anchored Reconstitution-Activated Proteins Highlight Intercellular Contacts (GRAPHIC), a novel synaptic tracing technology, to assess cortico-cortical connectivity. We found increased interhemispheric connectivity between Adnp deficient layer 2/3 pyramidal neurons in opposing somatosensory cortices. To probe the molecular mechanism of changes
to neuronal morphology, we performed localization analysis of Adnp as neurons underwent neurite formation. We found that Adnp is shuttled from the nucleus to the cytoplasm and this shuttling can be blocked via application of 14-3-3 inhibitor, difopein. Furthermore, we found that Adnp binds nuclear-cytoplasmic shuttle 14-3-3ε. We conclude that upon neuritogenesis, Adnp is shuttled from the nucleus to the cytoplasm by 14-3-3ε, where it regulates neurite formation, maturation, and functional cortical connectivity.

P1218

Guiding Neuronal Growth Cones With Light: A Computational Simulation Experiment
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During neural development, a sensory-motor structure called a growth cone extends from the axon of a neuron and navigates to a neighboring nerve cell, guided by various chemical and physical signals from its cellular environment. In vitro experimentation has demonstrated that optical signals provided by near-infrared (NIR) lasers are effective for guiding growth cone navigation, yet the underlying mechanism is not well established. We have developed a theoretical model to investigate how a NIR laser aimed at the growth cone’s leading edge induces growth cone turning. Our leading hypothesis is that the attractive force induced by the NIR laser biases the diffusion of globular actin proteins (G-actin) from the cellular environment towards the center of the beam, promoting rapid polymerization of the filamentous actin (F-actin) network near the site of the laser. We run computer-simulated experiments in MATLAB to investigate the effects of laser-induced asymmetric G-actin distributions on leading edge F-actin polymerization and leading edge protrusion. Our computational experiments predict the growth cone’s spatial reorganization, shape changes, and initial direction of motility in response to the NIR laser. Our simulations explore two scenarios for laser application, one in which the NIR laser’s position remains static and a second scenario in which the NIR laser is manually ‘steered’ in response to the protruding growth cone. Although our model does not incorporate axon mechanics, studies have shown that the neuron follows the direction of growth cone movement at some lesser angle. By developing a computational tool for predicting laser-induced growth cone turning, we have addressed an essential first step for applying NIR-based treatment to neurodegenerative diseases.

P1219

Glutathione-S-transferase Pi (Gstp) 1 and 2 regulate neurite formation in vitro and in vivo
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After neurons reach the cortical plate in the developing cerebral cortex, they start polarizing and extending neurites. Neurite initiation and elongation compose the process of neurite formation. This is crucial because it the basis of neurons building mature structures and forming networks with each other. Maldevelopment of neuron structure in early developmental stages can lead to intellectual defects and seizures in childhood. Gstp family proteins are enzymes involved in reductive reactions, and therefore have an essential role in protect from oxidative stress. Besides the enzymatic activity, Gstp proteins are also involved in cellular signaling. Studies on Gstp have mostly focused on its enzymatic
functions, such as in cancer. However, little is known for the functions of Gstp proteins in brain development. In our study, we found that mouse Gstp proteins play important roles in the neurite formation. Knockdown of mouse Gstp1 and 2 in primary cortical neurons using shRNA caused a significant decrease in neurite number at DIV2 after re-plating. In utero electroporation at E15.5 enables us to label layer 2/3 cortical neurons and analyze neurite formation in vivo. We combined in utero electroporation with ex vivo live imaging on brain slices. We found the Gstp 1 and 2 knockdown neurons resulted in defects in apical dendrite formation at P0, as the apical dendrite tips turned more frequently, and had fewer retraction events compared to the control neurons. The morphological defects persist to later developmental stages, including widening of apical dendrite at P3 and decreased basal dendrite number in P15. Furthermore, using a c-Jun N terminal kinase (JNK) inhibitor in primary cortical neurons, we confirmed the morphological defect caused by Gstp 1 and 2 knockdown was rescued, suggesting the involvement of Gstp 1 and 2 in the neurite formation process through JNK pathway. Based on our observations, we conclude that the Gstp1 and 2 are involved in neurite formation, especially important in the neurite initiation stage. Since the JNK pathway are well known as important regulator in neurite formation and microtubule organization, and the JNK pathway is often activated under stress condition, the results attract us to further investigate the roles of Gstp/JNK pathway and microtubule organization in neurite formation with and without stress condition.

P1220

Planar cell polarity proteins in neuronal polarity and AIS onset

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The axonal initial segment (AIS) is a neuronal subdomain that control the axo-dendritic sorting and the maintenance of neuronal polarity, and constitutes the site for action potential tuning. The AIS structure relies on a highly organized cytoskeleton and associated molecules like Ankyrin-G (AnkG) and βIV spectrin. AnkG is the AIS master organizer, since its early arrival at the proximal axon leads to the recruitment of all the other AIS components. While we know of a number of AIS proteins necessary for AnkG/AIS maintenance, the earliest steps of AIS assembly, including AnkG recruitment and anchoring at the membrane are still elusive, probably because of missing early AnkG partners. We found that some PCP proteins accumulate at the AIS at the onset of neuronal polarity, with specific colocalization with the main AIS members, especially AnkG and βIV spectrin, and confirmed direct interactions. Downregulation of these PCP genes impaired AIS formation, including the distribution and levels of the main AIS components, and also affected cytoskeleton organization. Finally, we show that some of the AIS functions were also affected, notably cytoplasmic filtering. Our study reveals a new role for PCP proteins as new players in neuronal polarity and function.
Mechanism of Gβγ-dependent regulation of neuronal differentiation and neurodegeneration

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BACKGROUND: Neurodegeneration is a pathological condition associated with progressive loss/dysfunction of neurons and occurs in many neurological disorders including Alzheimer’s disease (AD), Parkinson’s disease (PD), Huntington Disease (HD), amyotrophic lateral sclerosis (ALS), and dementia. Disruption of microtubules (MTs) in neurons, hyperphosphorylation of MT-associated protein (MAP) tau, and the aggregation of proteins including hyperphosphorylated tau proteins are hallmarks of neurodegeneration. However, the cause of this disorder is largely unknown, no effective drugs are available to treat the disease. Previous results from our laboratory have shown that Gβγ, an important component of GPCR pathway promotes microtubule (MT) assembly and induces neuronal differentiation of PC12 cells and blocking the interaction between Gβγ and tubulin/MT, inhibited neurite outgrowth, and induced axonal damage indicating the possible involvement of Gβγ in the process.

GOAL: In the current investigation we used human neuronal cells SHSY5Y to further understand the mechanism by which Gβγ is involved in neuronal differentiation and neurodegeneration. The involvement of phosphorylation of tau and GSK3β (a key molecule in Gβγ/PI3K signaling pathway) in this process was also elucidated.

METHODS: Gallein (GAL), an inhibitor of Gβγ, known to bind to Gβγ with high affinity and block the Gβγ dependent cellular activities, was used in this study. Both SHSY5Y and retinoic Acid (RA) differentiated SHSY5Y cells were treated with 10μM GAL for 1h, and subjected to whole cell lysis, co-immunoprecipitation (Co-IP) and confocal-scanning microscopy.

RESULTS: Microscopic analysis revealed that morphology of both SHSY5Y cells and SHSY5Y cells treated with RA were altered in the presence of GAL, Neurites appeared thinner and shorter when compared to the control cells. Co-IP analysis reveals that GAL inhibits the interaction between tubulin and Gβγ. Tubulin-Tau interaction which is important for MT assembly/neurite outgrowth was also inhibited in the presence GAL. Immunoblot using p359 TAU specific antibody showed that the expression of phosphorylated tau is decreased in the presence of GAL. In addition, GAL blocked phosphorylation of GSK3β, critical for MT assembly and neurite outgrowth.

CONCLUSION: The results indicate that GAL interferes with tubulin-Gβγ interactions, tubulin-tau interactions, and phosphorylation of GSK3β, the events are important for MT assembly/neurite outgrowth. Tau phosphorylation in the presence of GAL needs to be further investigated with additional phosphorylation-specific antibodies to tau. Thus, the results obtained from this study could provide a mechanism by which Gβγ is involved in neuronal differentiation and possible neurodegeneration.

Rpsa signaling regulates cortical neuronal morphogenesis via its ligand, PEDF, and plasma membrane interaction partner, Itga6

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All stages of neuromorphogenesis must occur with fidelity to ensure proper function of a mature neuron, with deficits in neuronal morphology implicated in the etiology of neurodevelopmental disorders. To understand how a signaling mechanism regulates neuromorphogenesis, several
developmental stages must be investigated and defects at any stage could directly impact subsequent stages, leading to multiple levels of defects. We identified a function for ribosomal protein SA (Rpsa) in regulating neuromorphogenesis using in utero electroporation to knockdown Rpsa, which results in apical dendrite misorientation at P3 and fewer/shorter extensions with decreased arborization, and decreased spine density with altered spine morphology at P15. To further delineate the signaling mechanism, we investigated Rpsa’s interacting partner on the plasma membrane, Integrin subunit α6 (Itga6), and its ligand, pigment epithelium-derived factor (PEDF). Serpinf1, which is the gene for the PEDF protein, is encoded in a clinically relevant region of chromosome 17p13.3 known as the Miller-Dieker Syndrome critical region that is frequently deleted or duplicated in a variety of neurodevelopmental disorders. Therefore, investigating the relationship between PEDF and its receptor Rpsa in the developing cortex is of interest. Knockdown of Itga6 and PEDF via in utero electroporation revealed phenotypes similar to Rpsa knockdown, with Rpsa and Itga6 overexpression rescuing neurite formation and branching defects in PEDF deficient neurons in vivo. Taken together, these data suggest that PEDF is the ligand responsible for initiating Rpsa signaling to regulate neuronal morphogenesis. Additionally, Itga6 overexpression increases both the expression level and time spent by Rpsa in the plasma membrane by preventing ubiquitination of Rpsa, which would lead to internalization or degradation of Rpsa. Interestingly, knockdown of Itga6 did not lead to deficits in dendritic spine density or morphology, while knockdown of both PEDF and Rpsa caused decreased spine density and a clear shift towards more immature spine morphology. Since spine morphology is related to function, GCaMP6s was used to functionally analyze the effects of Rpsa knockdown via ex vivo calcium imaging. Rpsa deficient neurons showed less fluctuation in fluorescence intensity, suggesting defective subthreshold spontaneous calcium signaling. Our study identifies a role for PEDF-Rpsa-Itga6 signaling in regulating functionally relevant aspects of neuromorphogenesis, and further analysis of these molecules may yield insights into potential therapeutic candidates for neurodevelopmental disorders such as Miller-Dieker syndrome.

P1223

Completion of neuronal remodeling prompts myelination along developing motor axon branches

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Neuronal remodeling and myelination are two fundamental processes during neurodevelopment. How they influence each other remains largely unknown, even though their coordinated execution is critical for circuit function and often disrupted in neuropsychiatric disorders. The onset of myelination correlates in many axonal populations with cessation of developmental plasticity, during which
excessive branches are removed. Whether myelination stabilizes axon branches during remodeling or ongoing remodeling delays myelination is unclear. To study the interaction of synapse elimination and myelination we used the developing mouse neuromuscular junction. By modulating synaptic transmission, cytoskeletal dynamics and axonal transport in mouse motor axons, we show that local axon remodeling delays myelination onset and node formation. Conversely, glial differentiation does not determine outcome of axon remodeling. FRAP analysis on transgenic mice expressing GFP-tagged nodal proteins refuted the idea that delayed myelination is due to insufficient supply of structural components of the axon-glia unit. Rather, this delay is due to limited transport of promyelinating factors that appear to initiate myelination in a branch specific manner. Once the local cytoskeleton matures in axon branches that have completed competition, promyelinating factors are delivered in increasing amounts and local myelination is initiated. Indeed, we find increased delivery of overexpressed neuregulin into axon branches that have completed remodeling compared to branches that are still competing for synaptic territory. Thus, our study reveals an axon branch-specific fine-tuning mechanism that uses cytoskeletal maturation to locally coordinate axon remodeling and myelination.

NPC assembly

P1224

Torsin suppresses the nuclear envelope NEP1R1-CTDNEP1/ Lipin lipid metabolism pathway for nuclear pore complex biogenesis

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Torsins broadly affect the cell from inside the endoplasmic reticulum and nuclear envelope lumen by an unclear mechanism. This ranges from cell growth to nuclear pore biogenesis. Torsin loss is also associated with a spectrum of human neurological diseases. Here a fly mini-screen identifies that the diverse cellular functions of Torsins are explained by Torsin-mediated regulation of the NEP1R1-CTDNEP1 / Lipin PA phosphatase cascade. Further, this requires Torsin homo-oligomerization rather than ATPase activity. Torsin is especially active at the NE where it suppresses TAG production, removes CTDNEP1, and excludes Lipin from the nucleus. Moreover, elevated NEP1R1-CTDNEP1 activity blocks membrane remodeling and nucleoporin recruitment for interphase NPC biogenesis, thus explaining the importance of NE-localized pathway suppression. We conclude that a Torsin / NEP1R1-CTDNEP1/ Lipin pathway connects the ER/NE lumen with PA metabolism. This has remarkably broad effects on the cell, including previously unrecognized impact on nuclear pore biogenesis.

P1225

TorsinA and neuronal nuclear pore complex biogenesis.

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Nuclear pore complexes (NPCs) are large protein complexes composed of hundreds of nucleoporins that mediate nucleocytoplasmic transport of protein and RNA. NPC abnormalities are implicated in DYT1
dystonia, a neurodevelopmental movement disorder caused by a loss-of-function mutation in the gene encoding torsinA, a AAA+ protein localized to the endoplasmic reticular (ER)/nuclear envelope (NE) endomembrane space. Despite increasing evidence implicating NPC dysfunction in DYT1 and other neurological diseases, NPC biogenesis in neurons remains poorly understood. Furthermore, the biological function of torsinA and the molecular defects underlying DYT1 dystonia remain largely unknown. In mouse primary neurons, we find a steady upregulation in NPC biogenesis during neuronal maturation. While NPCs are uniformly distributed in wild-type neurons, torsinA-null neurons develop mislocalized clusters of NPCs that become increasingly severe as neurons mature. These clusters demonstrate features of halted intermediate states of NPC assembly, as they contain early- but not late-recruited NPC components. In contrast to the drastic difference in NPC distribution, NPC density is unaffected in torsinA-null neurons, suggesting that torsinA helps maintain NPC localization and assembly, but not number. Interestingly, induced knockout of torsinA past the window of neuronal NPC biogenesis does not impair NPC distribution, suggesting a crucial role for torsinA during a critical developmental window. Similar to prior findings in vivo, primary cultures of torsinA-null neurons develop evaginations of the inner nuclear membrane (NE buds). The emergence of NE buds in primary neurons coincides with the formation of mislocalized NPC clusters, thereby implicating an association between these events. Taken together, our findings suggest a novel function of torsinA in the localization and assembly of new NPCs during a key period of neuronal development, and implicate aberrant NPC biogenesis in the pathogenesis of DYT1 dystonia.

P1226

Nuclear envelope-vacuole interactions mitigate stress induced by nuclear pore complex assembly defects
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Cellular adaptations to different kinds of stress is one of the defining features of all life. Due to the inherent complexities associated with interphase nuclear pore complex (NPC) biogenesis into the nuclear envelope (NE), NPC assembly defects can occur that induce stress on different aspects of cellular function. Work to date establishes that nuclear transport and NE integrity are frequently disrupted when NPC assembly is compromised, but there is a critical information gap about if and how cells are able to respond to sustained stress caused by NPC biogenesis defects. Using widefield fluorescence and electron microscopy for analysis of several mutants in the budding yeast *Saccharomyces cerevisiae* that cause NPC assembly defects as models, we found that interorganelle contacts between the NE and vacuoles (yeast functional equivalent to lysosomes) expand in response to NPC assembly stress. These contact sites were enriched for proteins that are known to mediate nucleus-vacuole junctions (NVJs), as well as lipid droplets. Importantly, double mutant analyses revealed that NE-vacuole contacts cooperate with lipid droplets to maintain cellular viability and stabilize NPC formation in strains where NPC assembly is already compromised, demonstrating these contacts play essential roles in modulating stress. Furthermore, using fluorescence microscopy and western blots, we discovered that NVJs function with autophagy factors in different NPC assembly mutants to remodel the NE and degrade specific NPC proteins in vacuoles. Collectively, our work reveals that interorganelle contacts between the NE and vacuole perform novel functions to ameliorate NPC assembly stress. Given the essential roles NPCs play in nucleocytoplasmic transport, neurological function, and aging, our ongoing studies are aimed at
further defining factors that promote expansion of NE-vacuole interactions when NPC biogenesis is disrupted.

P1227

_Conserved inner nuclear membrane proteins regulate nuclear pore complex distribution in Schizosaccharomyces pombe_

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Nuclear pore complexes (NPCs) are a conserved feature of the eukaryotic nuclear envelope that facilitate transport between the nucleoplasm and cytoplasm. In metazoans, interactions with nuclear lamina anchor NPCs and control their distribution in the NE. Subregions of the NE enriched for A-type lamins and the inner nuclear membrane protein emerin have significantly lower NPC density, showing that NPC distribution is locally influenced by the composition of the inner nuclear membrane. Interestingly, heterogeneity in NPC distribution has also been reported in the fungi, which lack lamins. This suggests that lamin-independent mechanisms exist that influence NPC distribution and density, although these putative factors remain unknown. To address this question, we used 3D structured illumination microscopy to quantitatively assess NPC number and distribution in the fission yeast _Schizosaccharomyces pombe_. These studies revealed that _S. pombe_ maintains a constant NPC density throughout the cell cycle and following acute perturbations to nuclear size. NPCs are non-randomly distributed in the NE, and their distribution was unaltered by loss of heterochromatin. However, we observed both global and local changes to NPC distribution in strains lacking the conserved inner nuclear membrane proteins Lem2 and Ima1. Lem2, in particular, was specifically required to establish a zone of NPC exclusion surrounding the fungal centrosome equivalent, the spindle pole body. These studies establish a framework for future studies using _S. pombe_ to identify and quantify lamin-independent mechanisms that control NPC number and distribution in living cells.

P1228

_The coordinated assembly of a functional nuclear envelope after mitosis requires the transmembrane nucleoporin Ndc1_

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During nuclear envelope (NE) reformation after open mitosis, nuclear pore complex (NPCs) components assemble onto the surface of chromatin to form a transport competent nucleus. Recruitment of scaffold nucleoporins by the chromatin-associated protein MEL-28/Elys initiates NPC assembly. Scaffold nucleoporins form stable interactions with inner ring subcomplexes of the nuclear pore that associate with the nuclear membrane to form a nascent NPC. Incorporation of central FG repeat-nucleoporins generates a selective diffusion barrier to complete NPC assembly. Here, we use _C. elegans_ to show that highly conserved transmembrane nucleoporin Ndc1 enables the stable incorporation of scaffold nucleoporins into nascent nuclear pores during NE formation. Loss of Ndc1 results in reduced global levels of MEL-28 as well as all scaffold nucleoporins tested, a delay in the onset of nuclear transport and a reduced rate of nuclear expansion. While the scaffold nucleoporin Nup160::GFP in control NEs is highly immobile, loss of Ndc1 more than doubled the mobile faction of Nup160::GFP at the NE. These
data suggest that Ndc1 controls the stable incorporation of scaffold nucleoporins to drive post-mitotic nuclear pore complex assembly - unincorporated scaffold nucleoporins in Ndc1-depleted embryos may be highly unstable leading to the reduction of their overall global levels. Supporting a role for Ndc1 in post-mitotic NPC assembly, Ndc1 tagged with GFP at the endogenous locus by genome editing is recruited to the reforming NE following chromosome segregation and enriched in regions of the NE that coincide with NPC assembly. Together, our data suggest that Ndc1 controls the rate-limiting step in NPC assembly to coordinate the assembly and growth of a functional nuclear envelope during nuclear reformation.

P1229

**The reticulon homology domain-protein REEP4 is recruited to the inner nuclear membrane by ELYS and promotes nuclear pore complex formation**

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Nuclear pore complexes (NPCs) are channels for nucleocytoplasmic transport within the nuclear envelope. About half of a cell’s NPCs assemble into the closed nuclear envelope during interphase. The remaining half are re-constructed concomitantly with nuclear envelope reformation during anaphase in a process called post-mitotic NPC assembly. Post-mitotic NPC assembly is initiated by the NPC biogenesis factor ELYS. NPCs assemble at nuclear pores, which are sites of fusion of inner and outer nuclear membrane and constitute domains of high membrane curvature within the otherwise flat nuclear envelope. Both, interphase and post-mitotic NPC biogenesis routes thus require local deformation of membrane. Yet, the factors that control proper membrane remodeling for post-mitotic NPC assembly are unknown. Reticulon homology domain-proteins are known to create high curvature within the cytoplasmic endoplasmic reticulum (ER) and were so far found excluded from the nuclear envelope. Here, we report that the reticulon homology domain-protein REEP4 localizes not only to high-curvature membrane of the cytoplasmic ER but also to the inner nuclear membrane. REEP4 is recruited to the inner nuclear membrane by the NPC biogenesis factor ELYS and promotes NPC assembly. Our findings indicate that REEP4 and ELYS cooperate in NPC assembly during anaphase and suggest an unexpected role of REEP4 in coordinating nuclear envelope reformation with post-mitotic NPC biogenesis.

P1230

**Cul3 Substrate Adaptor SPOP Regulates the Nuclear Pore Protein NupJ**

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Cell processes like growth and division are tightly regulated. One such mechanism of regulation is ubiquitination. Ubiquitination can change a protein’s localization or activity, or it can mark the protein for degradation by the ubiquitin proteasome system. The final step of ubiquitination, transferring ubiquitin to the target protein, is mediated by E3 ligases and their substrate adaptors, proteins that allow E3 ligases to be selective in choosing their targets. Understanding the targets of E3 ligases and substrate adaptors, then, is crucial to understanding cell regulation and disease mechanisms linked to
misregulation of protein levels and activity. SPOP is a Cul3 E3 ligase substrate adaptor whose targets, such as c-Myc, PD-L1, and ERG, are crucial for cell cycle progression and cancer proliferation. Through a mass spectrometry screen, we identified SPOP as a potential regulator of NupJ, a nuclear pore protein. Overexpression of SPOP in HeLa cells leads to decreased protein levels of NupJ via immunoblotting, and SPOP and NupJ both co-localize at the nuclear envelope via immunofluorescence microscopy. Moreover, co-immunoprecipitation assays demonstrate that SPOP and NupJ bind to each other in vitro. Similar to overexpression of NupJ, siRNA against SPOP leads to an increase in the number of nuclear envelope defects. Overexpressed NupJ leads to defects in cell division. Our results suggest that SPOP targets NupJ for ubiquitin-mediated proteasomal degradation.

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P1231

Cell cycle-dependent active stress drives epithelia remodeling

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Epithelia have distinct cellular architectures, which are established in development, re-established after wounding, and maintained during tissue homeostasis despite cell turnover and mechanical perturbations. In turn, cell shape also controls tissue function as a regulator of cell differentiation, proliferation, and motility. Here we investigate cell shape changes in a model epithelial monolayer. After the onset of confluence, cells continue to proliferate and change shape over time, eventually leading to a final architecture characterized by arrested motion and more regular cell shapes. Such monolayer remodeling is robust, with qualitatively similar changes in cell shape and dynamics observed across disparate perturbations. Here we quantify differences in monolayer remodeling guided by the active vertex model to identify underlying order parameters controlling epithelial architecture. For instance, for monolayers formed atop extracellular matrix with varied stiffness, we find the cell density at which motion arrests varies significantly but the cell shape remains constant. In contrast, pharmacological perturbations can significantly alter the cell shape at which tissue dynamics is arrested. Remarkably, we find across all experimental conditions the final cell shape is well correlated to the cell proliferation rate. Furthermore, inhibition of the cell cycle immediately arrests both cell motility and shape change, demonstrating that active stress from cell cycle-dependent processes contributes significantly to monolayer remodeling. Thus, the architecture and mechanics of epithelial tissue can arise from an interplay between cell mechanics and stresses arising from cell cycle dynamics.

P1232

Flexocytes—an active-matter model that shows properties of cell motility and shape

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Information processing and stimulus sensitivity are key features of many motile cells and bacteria. For example, mesenchymal cells navigate by sensing their environment's rigidity, and bacteria adjust their propulsion based on their local density. Interestingly, even for mobile rigid confinements that contain self-propelled particles, the internal degrees of freedom can process mechanical stimuli and lead to

P1233

Characterisation of the mechnano-chemical relationship between actin and microtubule cytoskeletons and the active motion of cytoplasmic components

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Using the Drosophila oocyte as a model, we are studying the biophysical properties of cytoskeletons in vivo, how they generate the motion of cytoplasmic components, and how these motions impact back on cytoskeleton organisation. The distribution of cytoplasmic content requires various types of motions, including passive diffusion, active-direct transport and advection (created by cytoplasmic flows). However, whether specific membrane-bound organelles display selected motilities, and how each type of motion is related to the cytoskeleton or to precise organelle features is unknown. By using Differential Dynamic Microscopy, our first motion characterisation showed that both vesicles detected by Differential Interference Contrast (DIC) and a specialised cytoplasmic F-actin mesh move in a ballistic manner by advection, and by active diffusion. The absence of microtubules leads to absence of flows, preventing motion of these structures by advection, while absence of the actin mesh diminishes diffusion-dependent motion. Additionally, the behaviour of F-actin fits the advection-diffusion model applied to vesicle motion. However, DIC vesicle motion presents an average behaviour, and do not allow us to differentiate which vesicle populations is being observed. To test whether specific membrane-bound organelles display selected motilities, we focus on Rab5 and Rab7 endosomes, chosen based on our preliminary data suggesting a functional link with the actin mesh. We find that the motion of these populations comprises both advective and diffusive forces, although the extent to which a specific cytoskeleton influences their motion differs. Rab5 endosomes display higher diffusivity compared to Rab7. We also found that mitochondria display different motility compared to DIC vesicles. This may be a result of energy demands or morphological differences. The processes of fusion and fission affect the morphology and distribution of mitochondria, as does ageing. Manipulating these processes, we find that fragmented mitochondria display higher flow speed compared to controls, while aged mitochondria display morphological abnormalities and a dip in flow speed and diffusivity. We now aim to develop a
holistic understanding of this system-environment interaction by analysing how motion of organelles change during ageing, the correlation between the position and size of membrane-bound organelles and the type of motion they display, and the biomechanical relationship between some membrane-bound organelles and the actin mesh biogenesis. To further contribute to this complex motion-cytoskeleton interaction, we will also decipher the molecular mechanisms responsible for our recent observation that the regime of flows also impacts back in the direction of plus-end growth.

P1234

**Measuring membrane stiffness in mcf-7 cells using optical tweezers**

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Metastatic cells have been shown to have softer membranes than non-metastatic cells due to the increased activity of the actin cytoskeleton. The physical properties of cell membranes determine, in part, how a cell behaves. A method which measures these physical properties will help to predict the behavior of cancer cells. Previously developed methods for these types of measurements often rely on expensive equipment and complicated calculations. The use of the optical tweezers in a biological context offers a more simple, precise, and non-invasive way of observing cell mechanotransduction. The objective of this study was to develop a method which uses optical tweezers to measure the stiffness of MCF-7 cell membranes. This was done by using a high-resolution camera to observe the position of a carboxylated microsphere. The standard deviation of the microsphere’s position was measured in the x and y directions; these measurements were inserted into the equation \( K = (3/2*K_B*T)/\langle x^2 \rangle \) where \( K \) was the stiffness in Newtons/meter, \( K_B \) was the Boltzmann constant, \( T \) was the temperature in Kelvin, and \( x \) was the standard deviation of the microsphere’s position. The optical trap stiffness was calculated powers 50, 100, 150, 200, 250, and 300 mW to obtain calibration curves of \( K_{Tx} = 2.35e-7P - 9.34e-6 \) (\( R^2 = 0.993 \)) and \( K_{Ty} = 1.31e-7P - 5.38e-6 \) (\( R^2 = 0.987 \)) where \( P \) was the laser power in mW and \( K \) was in N/m. Carboxylated microspheres were incubated with MCF-7 cells to facilitate their adhesion to the membrane. The microspheres were then trapped on the membrane at the same laser powers, and a stiffness value was calculated at each power to generate another set of curves which denoted the stiffness of the optical trap plus the stiffness of the membrane; these curves were given as \( K_{TMax} = 3.06e-7P + 2.72e-5 \) (\( R^2 = 0.753 \)) and \( K_{TMx} = 1.43e-7P + 3.24e-5 \) (\( R^2 = 0.846 \)). The y intercepts of these curves theoretically represented the stiffness of the membrane since this was where the laser power would be 0 mW. The Pythagorean Theorem was used to find an average membrane stiffness from the stiffness in the x and y directions. The stiffness of the membrane was found to be 6.38e-5 ± 6.99e-6 N/m. On average, there was a 4.34-fold change from the \( K_T \) curve to the \( K_{TMx} \) curve. Future experiments will entail treating cells with deoxycholic acid to increase membrane fluidity to serve as a negative control. Taken together, these experiments validate a robust method for measuring membrane stiffness using an optical trap.

P1235

**Extracellular matrix stiffness promotes DNA repair**

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Cells feel and respond to the physical properties of their microenvironment. It is well established that mechanotransduction regulates gene expression and therefore phenotypic outcomes. We asked if the stiffness of the extracellular matrix (ECM) regulates DNA repair, another key function of the genome. We addressed this question using organoid models of the normal mammary gland (acini), as well as mammary epithelial cells cultured on polyacrylamide (PA) gels of defined stiffness. We found that the response to DNA damaging drugs and ionizing radiation, measured by enumeration of DNA repair foci, was amplified in cell monolayers growing on stiff glass substrates compared to acini in soft ECM hydrogel. Analyses of cells on ECM-coated PA gels also revealed increased DNA repair foci density with increased stiffness. This effect could not be explained by cell cycle imbalance. Moreover, we confirmed that ionizing radiation caused similar levels of DNA breaks in soft and stiff conditions, pointing to mechanogenomic regulation of the DNA damage response. Our data further indicate increased homologous recombination and non-homologous end joining repair activities in stiff contexts. Cells on stiff ECM survived better after DNA damage induction. We are using normal human breast tissue for in vivo validation and will also present preliminary data on the mechanisms linking ECM stiffness and genome maintenance. Based on our findings, we propose that a mechano-stimulated DNA damage response in stiff tissue promotes survival of cells with DNA lesions at the expense of apoptosis, leading to the propagation of somatic mutations. This model may partly explain the elevated breast cancer risk associated with high mammographic density, which reflects elevated fibrillar collagen and stiff breast tissue.

P1236

Tumor mechanics and genetic change: a broadly applicable strategy with fluorescent proteins and gene editing

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Tumors that arise in stiff, solid tumors clearly show many more genetic changes than liquid tumors or tumors in soft tissues. However, linking biochemical factors to genetics is limited, by the fact that current sequencing methods require killing cells which prevents tracking a cell before, during, and after a genetic change. We have developed a new method to track genetic changes in living cells under diverse biochemical and biophysical stresses. Fluorescent protein sequences such as GFP are fused by gene editing to single alleles of constitutive genes in order to track when a chromosome is lost and how it proceeds and/or evolves afterwards as a heritable genetic defect. All constitutive gene fusions that are made with CRISPR/Cas9-mediated homology directed repair, with standard donor plasmid designs. To controllably induce chromosome mis-segregation, we first used inhibitors of the spindle assembly checkpoint and showed cells that lose fluorescence also show increased mis-segregation and micronuclei, linking genome instability to aneuploidy. Chromosome loss is also heritable. We have thus far identified constitutive genes on chromosomes 5, 9, and 12 in both cancer and non-cancer cell lines, all of which show utility as reporters and are confirmed by single-nucleotide polymorphism arrays among other genetic methods. Although some genes and loci are unsuitable, the reporter approach
shows promise with stressed cells and solid tumor xenografts, which should help elucidate biophysical factors such as tumor rigidity and stress that might cause genetic change.

**Rab GTPases**

**P1237**

**A feedback mechanism mediated by myosin-dependent apical accumulation of Rab11 vesicles reinforces apical constriction**

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During tissue morphogenesis, cell shape changes driven by mechanical forces often require active regulation of intracellular trafficking. It is not fully understood how mechanical stimuli influence intracellular trafficking and how such regulations impact tissue mechanics. To address these questions, we investigated the behavior of Rab GTPases, the master regulators of intracellular trafficking, during apical constriction-mediated mesoderm invagination in *Drosophila*. We found that during apical constriction, vesicle-like structures marked by Rab11, a recycling endosome marker, become enriched in the vicinity of apical myosin. The apical accumulation of Rab11 vesicles does not require endocytosis. Instead, it depends on vesicle transport along microtubules and is sensitive to disruption of myosin activity. Our quantifications further reveal that the transport is substantially biased towards the apical direction. Acute inhibition of myosin rapidly eliminates the directional bias of transport and inhibits apical accumulation of the vesicles, suggesting that myosin activity may promote apical enrichment of Rab11 vesicles by influencing the directionality of the transport. At the apical domain, Rab11 vesicles are moderately enriched around the adherens junctions, suggesting a role of these vesicles in regulating adherens junctions. Injection of dominant negative Rab11 proteins can acutely inhibit Rab11 vesicle accumulation, which does not obviously affect apical myosin accumulation but instead causes fragmented apical adherens junctions, altered spatial organization of apical myosin, frequent myosin breaks and a reduction in apical constriction rate. We propose that apical myosin-induced biased transport of Rab11 vesicles serves as a feedback mechanism to promote proper organization of the contractile machineries and thereby facilitate apical constriction.

**P1238**

**Optogenetic study of endosome trafficking pre-abscission**

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The final stage of cell division, termed abscission, is the scission of the cytokinetic bridge that separates the two daughter cells. The small GTPase, Rab11, is an important regulator of a dividing cell’s timely abscission where it mediates vesicle transport into and possibly out of the cytokinetic bridge. In these studies, the function of Rab11-associated membranes was characterized in vitro (HeLa cells) and in vivo (zebrafish Kupffer’s vesicle) using second generation optogenetic tools which acutely cluster Rab11-membranes at multiple spatiotemporal scales. Localization of Rab11 vesicles was altered at targeted locations and specific times during development to confirm the role of Rab11 in abscission in vitro and further characterize its undescribed role in abscission in vivo. Optogenetic clustering of Rab11-
associated vesicles resulted in an increase in abscission failure in both contexts. Strikingly, we found that CFTR (cystic fibrosis transmembrane conductance receptor) organization at the apical membrane employed Rab11-membranes during Kupffer’s vesicle morphogenesis. Our results show that the trafficking of Rab11-associated membranes during the late steps of cytokinesis is important for successful cell division and organ development.

P1239

Is the Arabidopsis SCD Complex an Evolutionarily Conserved Regulator of Exocytosis?
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Much of the subcellular-level organization of eukaryotes is maintained by trafficking of membranes and proteins between membrane-bound organelles and membranous structures. While trafficking principles and machinery identified through yeast and mammalian cell studies are conserved across eukaryotic lifeforms, an emerging concept posits that proteins present in the LECA, which have been lost in yeast and mammalian cells, are present in a wide distribution of other eukaryotic lifeforms. One such candidate is the Stomatal Cytokinesis Defective (SCD) complex, which has been demonstrated in Arabidopsis thaliana to function in exocytic trafficking necessary for cytokinesis and cell expansion. Genetic, biochemical, and live-cell imaging analyses indicate that the SCD complex activates RabE GTPase, a homolog of the yeast exocytic Rab, Sec4p. In contrast to Sec2p/Rabin8, the yeast and mammalian Sec4p/Rab8 GEFs, which are absent from plants, the SCD complex is a heterotrimer composed of SCD1, SCD2, and the Myosin Tail Homology I (MyTH1) protein. As the phylogenetic distribution of the SCD complex is yet to be established, we searched the proteomes of diverse eukaryotic species for sequence and structural-based orthologs of the individual SCD complex members. Based upon our initial bioinformatic analysis, we hypothesize that subunits of the SCD complex are widely present outside of plants, but not in Opisthokonts, and thus may need to be incorporated in general models of the exocytic pathway in eukaryotes. To identify if SCD complex function is evolutionarily conserved, we utilized the moss, Physcomitrium patens, a non-vascular extant representative of the earliest land plants and assessed whether the moss SCD1 ortholog, PpSCD1, is required for cytokinesis and polarized trafficking during the protonemal growth stage. RNAi mediated silencing of PpSCD1 resulted in a severe reduction in plant size and protonemal branching in a pattern resembling phenotypes associated with cell division and expansion defects. The functions of the remaining SCD complex subunits are not yet defined in either plant systems. MyTH1, named for its structural homology to the tail region of class I myosin proteins, a myosin family absent in land plants, was identified through LC/MS-MS analysis, and whose association with SCD1 was validated via live cell imaging and pull-down assays. Preliminary studies indicate that AtMyTH1 is a phosphoinositide phosphate interactor, suggesting it recruits the SCD complex to membranes integral to exocytic trafficking. Functional characterization of myth1 Arabidopsis and Phsycomitrium lines is in progress, as is further functional characterization of the SCD complex in Physcomitrium.
P1240

**A RAB proximity ligation screen identifies novel potential RAB25 interacting proteins.**
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Vesicular transport is essential to maintain cellular functions and cell survival, and deregulation of various membrane trafficking regulators is associated with different diseases. One important class of membrane traffic regulators are the RAB GTPases. RABs are controlled by Guanine Exchange Factors (GEFs) that activate RABs by promoting the exchange of GDP for GTP, while GTPase Activating Proteins (GAPs) are responsible for inactivating them by inducing RABs intrinsic GTPase activity. The action of GEFs and GAPs allows to modulate the activity of RABs and recruit them to the right cellular compartments. Once activated, RABs recruit multiple effectors to mediate their functions. Thus, the identification of the regulators and interactors of RAB GTPases is essential to better understand the various mechanisms of membrane trafficking. In order to identify the different interactors of RABs, we have utilized a proximity ligation technique mediated by the APEX2 protein. This technique makes it possible to mark the protein environment associated with a RAB and thus to define proteins interacting directly or not with the RAB in question. To do this, we cloned and validated the expression of more than 30 RABs in HeLa cells, and subsequent proteomics studies were conducted. These analyses identified a large plethora of novel potential interactors, showing various degrees of specificity. Interestingly, several known GEFs, GAPs and effectors have been identified. From those, we observed that RAB25 was strongly associated with the protein Dedicator Of Cytokinesis 7 (DOCK7). RAB25 governs the recycling of receptors on the cell surface and the activation of signaling pathways controlling cell proliferation, cell migration and apoptosis. It is also known to have tumor suppressor properties or to act as an oncogene depending on the type of cancer. DOCK7 is a GEF mediating the activation of two Rho family GTPases, RAC1 and 3. Therefore, this suggests that DOCK7 could be a potential RAB25 effector and could modulate the activation of RAC1/3. In addition, a protein complex comprising NBEAL2, VAC14 and SEC16A that are known DOCK7 interactors was also observed in our proteomics analyses of RAB25. Thus, functional tests will be used to verify whether this complex is affected by the absence of RAB25.

P1241

**A new RAB21 negative regulator implicated in various membrane trafficking events**
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Membrane trafficking is an essential cellular homeostatic mechanism and as such, is tightly regulated. Membrane trafficking allows cells and organelles to exchange molecular components through vesicle transport. The family of RAB GTPase represents an important class of membrane trafficking regulators. Among these RABs, RAB21, a mostly early endosomal RAB, plays a central role coordinating sorting, recycling or degradation events of many proteins. RAB21 is implicated in the positive regulation of autophagy, in the recycling of EGFR and integrins and in multiple cargo sorting events along with the WASH complex. RAB21 itself, and as other RABs, is activated by Guanine Nucleotide Exchange Factors (GEFs), which exchange GDP for GTP, and deactivated by GTPase Activating Proteins (GAPs), which potentiate GTP hydrolysis. Three specific GEFs for RAB21 are described but surprisingly, no GAP were found to act on RAB21. Given the important roles played by RAB21 we aimed in this project to identify a
RAB21 GAP. To do so, we performed a genetic modifier screen in Drosophila. This allowed us to identify TBC1D25 as a potential negative regulator of RAB21. The interaction between these two was confirmed by co-immunoprecipitation and by proximity ligation assays (PLA). Directed mutagenesis was used to create an inactive TBC1D25 mutant (RK), where the catalytic arginine was replaced by a lysine to eliminate GAP activity. PLA experiments showed that TBC1D25-RK weakly interacts with RAB21, suggesting that TBC1D25 GAP activity was necessary to mediate the interaction with RAB21. Moreover, RAB21-TBC1D25 interaction was enhanced by inducing autophagy through starvation. Furthermore, in vivo experiments, in drosophila larval fat body showed an increase in the size and number of autophagosomes following TBC1D25 overexpression, phenocopying RAB21 depletion on autophagy. Moreover, TBC1D25 overexpression decreased clathrin-independent cargo sorting regulation, as observed with CD98, further phenocopying RAB21 loss of function. Finally, we tested the implication of TBC1D25 on B1 integrin trafficking. Immunofluorescences showed that TBC1D25 overexpression reduced B1 integrin localization at the plasma membrane, again mimicking RAB21 depletion. Biochemical GAP assays will be performed in order to specifically determine if TBC1D25 is a GAP for RAB21. Collectively, these results showed that TBC1D25 is a negative regulator of RAB21 and a potential RAB21 GAP. Elucidating RAB21 regulation will permit to better uncoupled its various roles in membrane trafficking events.

P1242

Rab-10 functions opposite of the agef-1/arf gtpase/ap-1 pathway to regulate vesicle trafficking
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Cell polarity is critical for the function of many cell types. In Caenorhabditis elegans, basolateral localization of the LET-23 Epidermal Growth Factor Receptor (EGFR) in the vulval precursor cells (VPCs) is required for the development of the vulva. A class I/II Arf GTPase pathway regulated by AGEF-1, a homolog of the mammalian BIG1/2 Arf GEFs, antagonizes EGFR signaling by promoting its apical trafficking. Loss of the AGEF-1/Arf GTPase/AP-1 pathway leads to increased basolateral EGFR localization and over induction of vulva tissue. Thus, this pathway is required to maintain the proper levels of EGFR at the basolateral membrane to ensure proper vulva induction. We found that RAB-10 GTPase has novel antagonistic interactions with the Arf GTPase pathway. RAB-10, AGEF-1 as well as the Arf GTPase pathway localize to Golgi and recycling endosomes to regulate polarized trafficking in epithelial cells, however, it is not known how their functions are coordinated. The focus of my project is to find how RAB-10 interacts with the Arf GTPase pathway and which effectors and regulators function with RAB-10 to regulate EGFR localization and signaling. RAB-10 alone is not required for normal EGFR signaling. However, it is required when ARF GTPase pathway activity is compromised in order to favor EGFR signaling. Our data shows that rab-10 mutants can suppress agef-1 mutants, but not arf-1 and ap-1. One possibility is that AGEF-1 actively inhibits RAB-10 and when AGEF-1 is absent and ARF GTPase pathway is disrupted, RAB-10 might interact with AP-1 to increase basolateral transportation of the EGFR receptor in order to increase EGFR signaling. Determining the effectors of RAB-10 in the regulation of EGFR signaling helps to better understand how RAB-10 promotes EGFR signaling as a novel regulator. Two RAB-10 effectors, CNT-1, an Arf6 GAP, and SEC-15, a subunit of the exocyst complex, are the potential RAB-10 effectors in the regulation of EGFR signaling. We found that CNT-1 functions with AGEF-1 opposite of
RAB-10 in the VPCs. It is possible that CNT-1 functions as part of the AGEF-1 pathway or in another pathway parallel to AGEF-1. Therefore, it is not the regulator of RAB-10 in its antagonistic function on AGEF-1 pathway and in general in the regulation of EGFR signaling.

P1243

Dynamic interactions of the *Toxoplasma* parasitophorous vacuole and mammalian Rab vesicles, sequestered within the PV with a possible role for host ESCRT-III components

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*Toxoplasma gondii* is an intracellular parasite that multiplies in the cytosol of mammalian cells within a parasitophorous vacuole (PV). *T. gondii* designs the PV membrane (PVM) by incorporating its own proteins, creating a specialized compartment that avoids fusion with mammalian organelles. Despite the nonfusogenic nature of the PV, we showed that *T. gondii* salvages lipids from various host organelles. How may the parasite obtain these nutrients? We showed that *Toxoplasma* diverts several mammalian Rab trafficking pathways to the PV and internalizes Rab vesicles into the PV. Host Rab vesicles penetrate into the PV via an IntraVacuolar Network (IVN) of membranous tubules secreted by the parasite, that fuse with the PVM. The intra-PV vesicles are then processed by a parasite lipase located to the IVN to release cargo. To provide a dynamic view of this process, we engineered a parasite strain expressing a fluorescently labeled protein TgGRA3 that localizes to the PVM and IVN. Using time-lapse fluorescence microscopy, we monitored interactions between the PV and host vesicles. In infected cells stably expressing GFP-Rab11A, GFP-Rab11A vesicles transiently contacted the PVM; some disembarked from the PVM and others were internalized in less than 20 sec. Intra-PV vesicles localized to the IVN and showed less and slower movement than in the host cytosol. GFP-Rab11A vesicles within IVN tubules accumulate in the middle of the PV, suggesting a dissociation from the PVM. We hypothesize that to mediate scission of IVN tubules from the PVM the parasite recruits host ESCRT-III components, involved in severing membranes. We infected HeLa cells transfected with either N- or C- terminally tagged CHMP4B. mEmerald-CHMP4B was distributed around the PV in structures reminiscent of “class E” compartments and as discrete puncta on the PVM and IVN, as expected if CHMP4B mediates scission of IVN tubules. In contrast, CHMP4B-mEmerald is predominantly distributed on the PVM. The more uniform distribution of CHMP4B-mEmerald on the PVM and absence on the IVN suggests that the C-term tag may interfere with later steps in scission. Thus, the two tagged versions of CHMP4B will be useful tools to identify parasite proteins involved in ESCRT-III activity at the PV, possibly uncovering mechanisms of cellular lipid transport.

P1244

Structural basis for Rab6 GTPase activation by the Ric1/Rgp1 complex

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Membrane trafficking is a highly coordinated process in eukaryotes essential for maintaining lipid homeostasis and sorting proteins to the proper location inside cells. At the hub of this dynamic set of pathways, the Golgi complex directs much of this sorting through vesicle mediated transport. Vesicles of
varying lipid composition loaded with different protein cargo are in constant flux through the Golgi and precise coordination of these processes requires communication between compartments. Rab GTPases are small signaling proteins that act as molecular switches to regulate vesicular trafficking. Rab6 functions at the Golgi and is important for endosome to Golgi retrograde transport in both yeast and metazoans and for anterograde exit from the TGN in metazoans. The Ric1/Rgp1 protein complex is a highly conserved guanine nucleotide exchange factor (GEF) required for activation of Rab6. In mammalian cells Ric1/Rgp1 is known to be regulated by the Rab33 GTPase, but it remains unresolved how the activity of the complex is regulated and how it interacts with Rab6. Furthermore, very little is known about the structure and domain organization of Ric1/Rgp1. We have used cryo-EM to determine the high-resolution structure of the yeast Ric1/Rgp1-Rab6(Ypt6) complex, representing the key intermediate of the nucleotide exchange reaction. This structure reveals the overall architecture of the complex and has enabled us to identify the specific interactions that govern activation of Rab6. Ongoing studies aim to test the physiological significance of these interactions and determine the orientation of this complex on the Golgi membrane surface.

P1245

Fast ER-to-Golgi transport of oligomeric SURF4 cargoes via tubular Rab1A carriers
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About 6000 different human proteins, after being synthesized in the endoplasmic reticulum (ER), are transported to the Golgi apparatus for secretion or sorting to other organelles. How cells selectively transport different proteins to fulfill their various functions has elicited a wealth of interest in the past three decades. Distinct cargo receptors on the ER membrane have been identified to transport different proteins to the Golgi apparatus. Although these receptors have been demonstrated to bind cargoes with specific sequences or structural features, the cell biological consequences of cargo selection, especially its effects on transport kinetics and the diversity of cargo carriers, remain largely unknown. Here we report a fast-moving tubular ER-to-Golgi carrier from a serendipitous observation of a mislocalized ER-targeting fluorescent protein in mammalian cells. The tubular carrier lacks the authentic ER-Golgi intermediate compartment (ERGIC) marker ERGIC-53 but is marked by the small GTPase Rab1A. We reveal the cargo receptor SURF4 as the specific mediator to this tubular carrier, which serves as an express ER-to-Golgi transport route by virtue of its fast-moving nature (>1 μm/sec). Using the Retention Using Selective Hooks (RUSH) assay, we show that the ER-to-Golgi transport of cargoes with N-terminal point mutations, which abolish their SURF4 binding, is slowed down by more than two-fold. SURF4 knockdown dramatically reduces the transport rate of its cargoes to a level comparable to the bulk flow mechanism. Utilizing a well-characterized panel of fluorescent proteins, we uncover the dependence of the SURF4-mediated fast ER-to-Golgi transport on the oligomerization state of the cargo. Oligomeric fluorescent proteins with SURF4-binding N-termini are enriched at the ER exit sites through liquid-liquid phase separation with SURF4, which primes their ER exit and the formation of Rab1A-positive tubular carriers. Monomeric or SURF4-incompatible cargoes accumulate in the ER at steady state with a low ER-to-Golgi flow rate. Our results demonstrate that specific cargo carriers can modulate the ER-to-Golgi transport kinetics by sorting different cargoes into distinct ERGIC subtypes. The N-termini of proteins synthesized in the ER lumen, which dictate their binding affinities to SURF4, are therefore a significant yet overlooked determinant of the ER-to-Golgi transport kinetics.
Intracellular Nanovesicles Mediate Integrin Trafficking During Cell Migration

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Membrane traffic is an important regulator of cell migration through the endocytosis and recycling of cell surface receptors such as integrin heterodimers. Intracellular nanovesicles (INVs), are a recently identified class of transport vesicle that are involved in multiple membrane trafficking steps, including the recycling pathway. To date, the only known marker for INVs is Tumor Protein D54 (TPD54/TPD52L2), a member of the TPDS2-like protein family. Overexpression of TPDS2-like family proteins in cancer has been linked to poor prognosis and an aggressive metastatic phenotype which suggests that cell migration may be altered under these conditions. Here we show that TPD54 associates with INVs by directly binding high curvature membrane via a positively-charged motif in its C-terminus. This motif is shared among TPDS2-like protein family members and conserved in metazoans. We show that other members of the TPDS2-like family are also associated with INVs, and we document the collective Rab GTPase complement of all INVs. INVs collectively have at least 15 Rabs associated with them, and all have at least one TPDS2-like protein. Depletion of TPDS2-like proteins inhibits cell migration and invasion; and we show that this is likely due to altered integrin recycling. Our study highlights the involvement of INVs in the trafficking of cell surface proteins to generate biologically important outputs in health and disease.

Regulation of Protein-Protein Interactions and Mechanical Tension

P1247

Spectrins stabilize extracellular and intracellular polymer networks to regulate endothelial shear responses

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Shear stress is a frictional force exerted on the vascular lumen by flowing blood. Endothelial cells in the vessel wall sense and respond to this force by secreting vasoactive compounds and by undergoing extensive cytoskeletal remodelling to orient themselves in a direction parallel to the shear axis. Previous work has identified that luminal glycopolymers are involved in shear-sensing and that shear responses require the rearrangement and activation of basolateral integrins. However, a unifying mechanism for how these directional signals are transmitted from the apical to the basolateral cell surface is lacking. We identified a luminal spectrin network in the endothelium that is required for normal cellular alignment along the shear axis. Using single molecule tracking in combination with super-resolution microscopy, we found that this network is required for the immobilization and apical retention of the transmembrane glycoprotein CD44. By stabilizing CD44, spectrin also regulated the surface density of hyaluronic acid, an abundant component of the endothelial glycocalyx that is required for shear-sensing. Interestingly, spectrin depletion resulted in a significant increase in the elastic modulus of endothelial cells as measured by atomic force microscopy. This suggested that spectrin also has a role in regulating the gross cytoskeletal composition of these cells. Importantly, we found that spectrin interacts with and
stabilizes actin and vimentin filaments involved in force transduction. Taken together, our observations support the notion that spectrins play a central role in stabilizing mechanosensory and mechanoresponsive machinery in endothelial cells to coordinate responses to shear stress.

P1248

**How Adherent Cells Reorient in Response to Uniaxial Cyclic Stretching**

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Many types of adherent cells are known to respond to uniaxial cyclic stretching by reorienting perpendicularly to the direction of stretching. While this phenomenon has been documented for decades, the underlying mechanism remains poorly understood. To address this question, we used a simple on-stage stretching approach that allows programmable stretching-relaxation and synchronized imaging of cells cultured on polyacrylamide substrates. We observed that the reorientation of NRK epithelial cells involved rapid shortening along the direction of stretching (referred to as longitudinal direction) and progressive elongation perpendicularly to the direction of stretching (referred to as lateral direction). By analyzing differences of images collected at the beginning and end of stretching or relaxation phases, we found that while retraction mainly occurred during the stretching phase, extension predominantly took place during the subsequent relaxation phase. Moreover, although both longitudinal and lateral directions show extensions during the relaxation phase, lateral extensions were persistent while longitudinal extensions decreased with time, which led to shape change. Retractions during stretching phase showed no detectable directional preference. Interestingly, disassembly of microtubules promoted cyclic-stretching-induced shape changes through a rapid partial inhibition of longitudinal extension followed by a slow stimulation of lateral extension. Together, these results imply a microtubule-mediated mechanism that regulates the long-range transport of retraction signals away from longitudinal ends during the stretching phase, which may in turn affect the generation of protrusion signals during the subsequent relaxation phase in a position and concentration-dependent manner. We proposed that cyclic-stretch-induced, microtubules-mediated cell reorientation is a result of decreasing longitudinal extension coupled with consistent lateral extension as a function of time during relaxation phase, which leads to shape changes of rapid longitudinal shortening and progressive lateral elongation.

P1249

**The multimodular lim domain protein testin recognizes stress fiber strain and contributes to cellular force generation**

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Cells are complex machines exhibiting a plethora of mechanical behaviors, including division, movement and growth, which contribute to tissue development and homeostasis. It has been shown that mechanical signals (e.g. pressure, stiffness) are just as capable as biochemical signals of affecting cellular behavior. Cells sense these mechanical signals and convert them into biochemical signals in a process collectively called mechanotransduction. However, the molecular mechanisms underlying this process remain poorly understood. The actin cytoskeleton, in conjunction with its associated proteins, is thought to play a key role in this process via both stress fibers (SF) and focal adhesions (FA). These structures
enable cells to generate forces and transmit them from the cytoskeleton to the extracellular environment or vice versa. The family of LIM domain-containing proteins (LDP) have emerged as important mechanoresponsive cytoskeletal proteins. For example, both LDPs zyxin and paxillin have been shown to localize to SF strain sites. To gain insight into LDP mechanosensitivity and their potential contribution to cellular force generation and transmission, we investigated testin, another LIM domain and cancer-associated protein. Testin consists of an N-terminal CR (cysteine rich), central PET and three C-terminal LIM domains. When truncated, the N-terminal domains (CR and PET) recognize SFs whereas the LIM domains localize to FAs. When we induce strain in SFs, only the LIM domains and not the N-terminal half, recognize these strain sites. Interestingly, unlike zyxin, an individual LIM domain is sufficient for mechanosensitivity. Only SFs that are not fully severed show LIM domain recruitment suggesting they recognize a strain-induced conformational change in the actin filaments and not free barbed ends. Full-length testin, in contrast, is distributed throughout the cytoplasm and not capable of relocating to SF strain sites. This observation is possibly explained by testin’s ability to dimerize which potentially blocks the accessibility of the LIM domains and suggests testin’s mechanosensitivity is regulated. Using point mutations of tyrosines 111 (PET) and 288 (LIM 1), regions thought to associate during dimerization, we show that testin becomes mechanosensitive, enabling it to recognize SF strain sites. In addition, two cancer-associated testin mutants (E185K and E306K) similarly relocate to SF strain sites. Using Traction Force Microscopy, we then found that these mutations promoted cellular contractility. Together, our results show that testin recognizes local SF strain and indicate its mechanosensitivity is regulated and potentially linked to its involvement in cancer.

P1250

Spatiotemporal control of talin-mediated mechanotransduction by inducible dimerization
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Talin is a key constituent of integrin-based focal adhesions (FAs). Talin serves as the structural and molecular backbone of FAs, physically connecting integrin with the actin cytoskeleton, and playing crucial roles in the sensing of the cell mechanical microenvironment. Characterisation of FAs by super-resolution microscopy and proteomics have revealed a complex hierarchy of structural and compositional organization. However, further mechanistic dissection of the dynamics of talin-mediated mechanotransduction remains challenging due to such complexity as well as the lack of precise molecule-specific control. In particular, although perturbations of force-generation and cell signalling by pharmacological or mechanical intervention are well established, the ability to control intramolecular force-transmission with molecular specificity has been relatively underdeveloped. To address this, we have generated a toolbox of engineered talin constructs in which chemically or optically inducible dimerizer modules such as FRB/FKBP, iLID, and LOVTRAP are inserted at specific sites between talin rod domains. These modules separate the key integrin and actin binding sites, thus allowing talin-mediated force transmission to be turned on by rapamycin, or turned on/off by blue light illumination, respectively. Using magnetic tweezer force spectroscopy, we showed that these dimerizer modules can support 5-10 pN force, thus withstanding tension being transmitted through talin in FAs. These highly modular constructs can be combinatorially applied to probe force transmission and functions of distinct talin domains. In talin-null background cells, we demonstrated that these switchable talins support FA formation, traction force generation, cell migration, as well as recapitulate actomyosin contractility-
dependent FA maturation. Finally, we showed that intra-molecular force transmission through talin is required for the nuclear translocation of the transcription factor YAP and subsequent mechanosensitive transcription. In conclusion, these tools provide a spatiotemporally precise and versatile approach for direct and molecule-specific control of mechanotransduction, compatible with live-cell imaging, and generalizable to application in other force-transmitting proteins.

P1251

Mechanosensitive Myosin Turnover Dynamics in Contractile Actomyosin Structures

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Dynamic cellular processes, such as cell migration and cell division, require mechanical forces generated by the actomyosin cytoskeleton, where myosin molecular motors pull on actin filaments (F-actin). In non-muscle cells, one of the major contractile actomyosin networks is the stress fiber. While stress fibers are structurally similar to sarcomeres in muscle cells, they are more dynamic as actin and myosin constantly turn over. Furthermore, stress fibers can quickly remodel upon mechanical perturbation, for example when cells are stretched or when cells migrate. However, the molecular mechanism remains unclear as to how actomyosin turnover contributes to stress fiber remodeling. In this work, we focus on the turnover of non-muscle myosin motors (hereby referred to as myosin), which can transition between its monomer and filament states to modulate tension. While myosin monomers are non-processive motors and thus incapable of producing force, monomers can self-assemble into bipolar minifilaments as the functional unit to exert force on F-actin. We hypothesize that the turnover of myosin between its two states allows stress fibers to remodel in response to mechanical perturbation. Therefore, to assess myosin dynamics on stress fibers, we conducted fluorescence recovery after photobleaching (FRAP) experiments on the transverse arcs of U2OS cells. With super-resolution live-cell imaging, we could see myosin bands on individual stress fibers recover after photobleaching. Interestingly, different myosin bands within a single stress fiber exhibit slight differences in recovery kinetics. To perturb cellular tension, cells are treated with the Rho-kinase inhibitor Y27632, where myosin filament assembly is inhibited. Consistent with past literature, myosin recovers much faster after photobleaching, which is thought to be due to an increase in diffusive myosin monomers. However, using a simple reaction-diffusion model, we find that our data is inconsistent with a diffusion-dominant FRAP profile. Instead, we hypothesize that the faster FRAP recovery is due to force-dependent reaction dynamics, i.e. reduced cellular tension leads to faster myosin turnover rates. To investigate this further, we modulate tension by washing out Y27632 to observe myosin dynamics as tension reestablishes. Interestingly, we find that myosin reassembles homogeneously throughout the transverse arc region instead of starting from the leading edge. Combined with super-resolution imaging, our data sheds light on the microscopic mechanism of how myosin turnover responds to cellular tension.
How does strain rate impact mechanosensitive cell division in stretched tissue?

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Cells in developing tissue must be able to cope with a variety of forces including tensile (stretching), compressive (pushing) and shearing (a combination of tensile and compressive forces) and it is vital that cells can respond appropriately to these forces. Mechanical regulation of cell division is an example of such a response, i.e. cells change their division rate and align division orientations in a manner that helps relieve mechanical stresses and maintain tissue homeostasis. Previous research has explored the effects of mechanical forces upon single cultured cells. However, much less is known about the response of cells to mechanical forces in complex tissues. To bridge this gap, we are using Xenopus laevis animal cap explants to investigate how mechanical force regulates cell behaviour in a multi-layered tissue. In previous work, we have shown that when animal caps are stretched, the superficial epithelial layer responds to tensile force by increasing cell division rate and reorienting divisions along the axis of stretch. However, what is unclear is how these cells respond to the same tensile force applied at different rates. This is particularly important as the instantaneous application of the tensile force widely seen in the literature is unlikely to replicate the conditions cells experience in vivo, for example during tissue morphogenesis, where a tensile strain is experienced over the course of many hours. A new discrete formulation of the popular vertex model is introduced, with the aim of providing a useful tool to investigate the mechanical environment and its effects on epithelial cells. Using this model we are able to show that an instantaneous application of strain causes more mechanical stress in a tissue than applying the same amount of strain over a longer period. Preliminary results show that cells subjected to the same force over a longer period do not experience an increase in proliferation rate, unlike the instantaneous cases seen in literature. However cells experiencing the same force do reorient divisions along the stretch axis, regardless of strain rate. These results indicate that cells use different mechanisms to control their proliferation rate and their division angle. Proliferation rate may be connected to the mechanical stress experienced by the cell, whereas division angle may be influenced greater by the strain experienced.

The role of Nuclear Mitotic Apparatus (NuMA) protein in orienting cell division in tissue under mechanical tension

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Cells within an organism constantly experience a variety of mechanical forces from their surrounding tissue environment. Reading and responding to these forces is crucial to normal development and errors in this process can contribute to failures in embryogenesis as well as diseases such as cancer. One of the cellular functions that forces impact is cell division, influencing both division rate and orientation. Division orientation is determined by the mitotic spindle, a structure that accurately segregates the cell’s genetic material between two identical daughter cells. To influence division orientation, forces must be perceived from the external environment and relayed to the mitotic spindle but this process remains
poorly characterised. Nuclear Mitotic Apparatus (NuMA) protein is known to be a crucial component of the cell’s spindle orientation machinery and has been recently implicated in orienting the spindle according to force. However, little is known about the mechanistic details of NuMA function in mechanosensitive spindle orientation, especially in a tissue context. To this end, we utilise the Xenopus laevis embryonic animal cap tissue, to which reproducible tensile forces can be externally applied, to investigate cell divisions and to understand the role of NuMA in mechanosensitive spindle orientation. Using expression of exogenous GFP-tagged human NuMA, we show that cortical localisation of NuMA is highly dynamic and sensitive to mechanical stretch, with recruitment to the polar cortex earlier during mitosis in stretched tissues. Furthermore, a mathematical model based on microtubule pulling, results in the mitotic spindle aligning with the discrete cortical localisation of NuMA prior to chromosome segregation. The direction of the modelled spindle best matches experiments when pulling forces are amplified at regions of localised NuMA as opposed to other possible pulling regimes. Using morpholino-targeted knockdown of endogenous Xenopus laevis NuMA expression in early embryos, we also show that knockdown of NuMA disrupts the ability of cells to orient mitotic divisions along the axis of mechanical stretch and cell shape. Overall, with a combination of live tissue imaging and mathematical modelling, these results indicate that NuMA is important for spindle orientation according to external force.

P1254

Biophysical properties controlling septin filament assembly

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Cells build micron-scaled structures from nanometer-scaled proteins. Septins are a highly conserved class of GTP-binding proteins that dynamically assemble into higher-order structures. Higher-order septin structures serve as platforms that regulate polymerization dynamics of other actin and microtubules, recruit proteins necessary cytokinesis, mediate local cell signaling, and organize the plasma membrane. Despite these newly appreciated roles for septins, very little is known about how cells control septin filament polymerization. Genetic and biochemical studies in yeast have identified a suite of septin regulators. Marrying a filament polymerization reconstitution system with cell extracts utilizing yeast genetics, we begin to investigate how regulators tune biophysical processes necessary to build higher-order septin assemblies. Filaments polymerized from extracts were paired and their flexibility could be tuned by regulators. Septin assembly is a multi-step process involving septin interactions with the membrane and between septin proteins themselves during filament polymerization. Whereas septin adsorption onto membranes is a cooperative process, multiple aspects of filament assembly on membranes are consistent with isodesmic filament polymerization. We parameterized a physical model of septin filament assembly on membranes laying the groundwork for future investigations into how regulators control assembly of higher-order septin structures.
Regulatory and Noncoding RNAs

P1255

An adherens junction-associated RISC suppresses distinct isoforms of the oncogenic MIR17HG lncRNA to maintain epithelial cell homeostasis

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The adherens junctions (AJs) are essential architectural elements of epithelial tissues. Recently, we identified a novel mechanism, whereby the AJ component, PLEKHA7, recruits the RNAi machinery, including RISC and its main component Ago2, as well as miRNAs, to suppress pro-tumorigenic cell transformation. Long non-coding RNAs (lncRNAs) can also interact with the RNAi machinery and have been implicated in tumorigenesis; however, the mechanisms directing lncRNA regulation remain largely unclear. We hypothesize that PLEKHA7 associates with and regulates lncRNAs via the AJ-localized RNAi machinery. Indeed, by performing PLEKHA7 RNA-CLIP and subsequent RNA-Seq analysis, we identified association with numerous lncRNAs. In addition, RNA-Seq in PLEKHA7-depleted colon epithelial Caco2 cells revealed differential expression of 49 of the AJ-associated lncRNAs, showing that PLEKHA7 also regulates their levels. Of these, the top upregulated lncRNA was MIR17HG, which has two alternatively spliced isoforms; the longer of these isoforms (MIR17HG_L) is a polycistronic host transcript of a set of miRNAs (miR-17-92 cluster), including the known oncogenic miR-19a and miR-19b miRNAs. However, regulation and function of the shorter MIR17HG isoform (MIR17HG_S) has been overlooked thus far. Our data show that PLEKHA7 depletion increases the levels of both MIR17HG isoforms and of miR-19a and b. Notably, MIR17HG_S is more strongly upregulated by PLEKHA7 depletion than the longer transcript. In agreement, qRT-PCR analysis and data mining from patient colon tumor samples show that MIR17HG_S is indeed the most abundant MIR17HG isoform and strongly upregulated in colon tumors. Data from Ago2 knockdown, antago-miR, and miRNA mimetic experiments show that PLEKHA7 suppresses the levels of the MIR17HG_S transcript through the AJ-associated RISC and miRNAs. In addition, uncoupling of Ago2 from AJs distinctly leads to an increase in MIR17HG_S levels, demonstrating that RISC localization to the AJs is required to suppress MIR17HG_S levels. We have also found extensive mis-localization or downregulation of PLEKHA7, co-existent with mis-localization of the junctional RNAi machinery, in colon cancer tissues and cell lines. Ectopic expression of PLEKHA7 in aggressive colon cancer cells that lack endogenous PLEKHA7 expression, suppressed elevated MIR17HG_S levels, as well as cell growth, in vitro and in vivo. In summary, our findings point towards a novel mechanism of localized lncRNA regulation through AJ-associated RNAi machinery that influences cell behavior. They also reveal a previously unappreciated lncRNA isoform with potential novel roles in colon tumorigenesis.
**P1256**

**Father knows best: Small RNA-mediated regulation of male fertility and paternal epigenetic inheritance in Caenorhabditis elegans**

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With diverse roles in essential biological processes such as development, genome stability, and fertility, small RNA (sRNA) pathways are key regulators of gene expression. sRNAs direct sequence-specific gene regulation by associating with effector Argonaute proteins (AGOs) to either degrade, inhibit translation, or promote expression of target transcripts. With a robust developmental program and an expanded group of 21 AGOs, *Caenorhabditis elegans* is a superb system to study these highly conserved regulatory mechanisms. Recently, our lab has systematically characterized the expression patterns and sRNA populations associated with all 21 AGOs in *C. elegans*. In doing so, we identified four AGOs with expression specific to the gonad during spermatogenesis, and nine expressed constitutively in the germline (germline constitutive). Although it is well established that sRNA pathways are essential for fertility in multiple organisms, the bulk of worm sRNA research has focused on roles in oogenesis. Therefore, we aim to understand how sRNA pathways contribute to proper sperm development and paternal epigenetic inheritance. By assessing trans-generational fertility in single and multiple spermatogenesis-specific ago mutants, we have observed reductions in fertility that can be rescued by mating to wild type males, pointing to defects in spermatogenesis. Our AGO sRNA binding data point to multiple facets of spermatogenesis and spermiogenesis that are regulated by distinct AGOs. Our current efforts are focused on understanding the molecular mechanisms by which the spermatogenesis AGOs contribute to various processes in the development and differentiation of fertile sperm. In addition to their genomic contribution, mature spermatids can carry several potential couriers of epigenetic information to progeny, including chromatin modifications, sRNAs and AGOs. Therefore, in parallel, we aim to understand which AGOs and sRNAs are passed from father to progeny via sperm. Using our complete set of GFP-tagged AGOs, we determined that only two AGOs are packaged into mature sperm. This finding has the potential to re-shape our understanding of paternal sRNA contributions to progeny. In sum, our work will define new pathways and gene regulatory modes that contribute to male fertility.

**P1257**

**A long non-coding RNA enhances intestinal epithelial antiparasitic defense through NF-κB p65 mediated transcriptional regulation of Irf7**

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The intestinal parasite *Cryptosporidium parvum* is a major cause of diarrheal disease worldwide, and is a significant AIDS-related opportunistic pathogen. Currently, no fully effective treatment exists. Long noncoding RNAs (lncRNAs) can act as key modulators of diverse cellular processes through interactions with DNA, RNA, and proteins. LncRNAs have been identified that play a role in the inflammatory response, however, the functional mechanism has been elucidated only in a select few. Previous work showed one lncRNA, referred to as lncRNA-25B, impacts the infection burden of *C. parvum* in the IEC4.1 cell line. Knockdown of lncRNA-25B using siRNA significantly increased the infection burden of *C. parvum*, while conversely, overexpression of lncRNA-25B significantly reduced the infection burden. To
understand how IncRNA-25B may be impacting infection burden, RNAseq was performed. Analysis of RNAseq data revealed dysregulation of numerous defense genes upon knockdown of IncRNA-25B. Interestingly, many of the impacted genes were NF-κB target genes, suggesting a potential role for IncRNA-25B in regulation of NF-κB signaling. RNA immunoprecipitation demonstrated a physical interaction between NF-κB p65 and IncRNA-25B, and that this interaction is significantly increased following C. parvum infection. Chromatin immunoprecipitation was employed to investigate whether IncRNA-25B impacts the localization of NF-κB p65 to defense genes. ChiP revealed a significant decrease in NF-κB p65 localization to the Irf7 promoter region upon knockdown of IncRNA-25B compared to a scrambled siRNA control. Chromatin isolation by RNA purification was used to determine whether IncRNA-25B specifically binds to the promoter region of Irf7. Upon C. parvum infection, there was a significantly increased interaction between IncRNA-25B and the promoter region of Irf7. To determine whether the regulation of Irf7 by IncRNA-25B is responsible for the differences observed in C. parvum infection burden, knockdown of Irf7 using siRNA was performed. The knockdown of Irf7 resulted in a significant increase in C. parvum infection burden compared to a scrambled siRNA. In conclusion, these experiments have identified a C. parvum induced IncRNA which was found to impact the parasite infection burden through interactions with NF-κB p65 and subsequent transcriptional regulation of the defense gene Irf7.

P1258

Synthetic guide rna for crispr-mediated transcriptional activation


The CRISPR-Cas9 system has been adapted for transcriptional activation (CRISPRa) and several second-generation CRISPRa systems (including VPR, SunTag, and SAM) have been developed to recruit multiple transcriptional activators to a transcriptional start site. Several studies have demonstrated CRISPRa in a pooled, lentiviral expression context but the use of synthetic CRISPR guide RNA for activation has not been reported. Here we show the effective use of synthetic guide RNA for gene activation with several second-generation CRISPRa systems. We demonstrate the use of CRISPRa crRNA with a canonical tracrRNA using the VPR system or with an extended tracrRNA containing an MS2 aptamer sequence for the SAM system. Using chemistries and processes developed over decades of research for the efficient, cost effective, and high throughput synthesis of guide RNAs over 100 nucleotides long, we tested adding either one or two MS2 aptamers to different regions of the tracrRNA in order to maximize gene expression with the SAM system. We find that transcriptional activation with a single MS2 aptamer in the synthetic crRNA:SAM tracrRNA complex is comparable to or higher than activation levels achieved from sgRNA expression vectors containing two MS2 aptamers. Furthermore, in both the VPR and SAM systems, combining several crRNA sequences targeting the same gene can enhance transcriptional activation levels, indicating the effectiveness of synthetic guide RNA for gain-of-function studies. Additionally, when working with cell types where viral transduction is not feasible, we show that transcriptional activation can be achieved in the VPR system using dCas9-VPR mRNA and synthetic crRNA:tracrRNA. We also demonstrate the use of synthetic guide RNA libraries in an arrayed CRISPR screening format, identifying both positive and negative regulators of the IL-6 cytokine. Together, these results and tools demonstrate the suitability of synthetic CRISPRa guide RNA for a variety of transcriptional activation applications, including genome-scale screens.
In silico and in vivo analysis of miR-181b-5p and miR-330-3p in visceral adipose tissue of obese individuals.

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Adipose tissue is key machinery in energy homeostasis, and increased visceral fat is a principal reason behind obesity and its associated metabolic complications. MicroRNAs (miRs or miRNAs) have been observed to play a role in the pathogenesis of obesity. Animal studies have reported dysregulated miR-181 and miR-330 in the development of obesity and insulin resistance. In this pilot experiment, we have demonstrated the expression levels of miR-181b-5p and miR-330-3p in human visceral adipose tissue (VAT) samples and predicted their target genes that contribute to the pathology of obesity. 11 obese and 22 non-obese control subjects, aged 18-60 years, and about to undergo abdominal laparoscopic surgery were recruited after taking informed consent. Fasting venous blood samples were collected for blood glucose, lipid profile, and insulin estimation. Insulin resistance indices were calculated from the biochemical parameters. Total RNA was extracted from freshly excised VAT samples taken during surgery. RNA was reverse transcribed, and quantitative PCR was carried out using RNU6 as an internal control. Target genes for the miR were predicted using miRWalk3.0, visualized through Cytoscape, and subsequently analyzed for Gene Ontology (GO) enrichment to identify the key pathways and processes involved. Statistical analyses were performed on R Studio. The two groups were significantly different in terms of body mass index (BMI) (p<0.001), waist-to-hip ratio (p=0.005), fasting blood glucose (p=0.002), HOMA-%β (p=0.006), HOMA2-%β (p<0.001), and Triglyceride-glucose index (p=0.006). There was a weak correlation of miR-330-3p with serum insulin (ρ=0.301, p=0.088) and BMI (p=0.343, p=0.051). Fold change expression revealed a 1.52 times upregulation for miR-181b-5p and a 3.13 times downregulation for miR-330-3p in obese subjects compared to non-obese individuals. 84 target genes with a score >0.90 and validated in miRTarBase, TargetScan, and miRDB were significantly involved in various Biological Processes such as cardiac fibroblast cell development, MAPKK activity activation, positive regulation of epithelial cell migration, and cell aging, Molecular Functions such as nuclear import and nucleic acid binding and Cellular Components. Further, genes responsible for insulin resistance and obesity, like MAP3K3, KPN1A, and DUSP5, and adipocyte differentiation such as PRX3, MAP3K10, and CPEB4 were predicted as targets for these two miRNA. The findings suggest that miR-181b-5p and miR-330-3p are associated with the development of obesity and may represent potential therapeutic markers.
attempt has been made at an in-silico analysis of miRNA and the role of GDF-15 in the development of IR and T2DM. We took three Gene Expression Omnibus (GEO) datasets, GSE9624, GSE29718, and GSE20950, and separated out the differentially expressed miRNA. The first two datasets are from obese populations, while the latter compares insulin-resistant individuals with insulin-sensitive individuals. The common miRNA between the datasets with a |logFC|≥0.58, i.e. fold change at least 1.5 times up- or downregulated, was miR-21-5p; and it was further experimentally tested in a cohort of 45 patients, equally divided into healthy controls, obese pre-diabetes, and diagnosed type 2 diabetics, all recruited after informed consent. Clinical history and anthropometric measurements were taken and the basic biochemical profile was carried out; insulin resistance indices were calculated using the homeostatic model assessment (HOMA) model. Circulating miR-21-5p and GDF-15 expression (housekeeping gene RNU6 and GAPDH, respectively) were evaluated by RT-qPCR. All statistical analyses were done using R software. The three groups were significantly different in terms of body-mass index (p<0.001), waist-hip ratio (p=0.001), HbA1c (p<0.001), fasting blood sugar (p<0.001), fasting insulin (p=0.014), HOMA-%β (p<0.001), total cholesterol (p=0.029), triglycerides (p=0.002), triglyceride-HDL ratio (p=0.009), and triglyceride glucose index (p<0.001). The expression of miR-21-5p was upregulated 2.58 and 5.70 times in prediabetic and T2DM patients, respectively, compared to healthy controls, while for GDF-15, the upregulation was 1.58 and 2.35 times, respectively. There was a strong positive correlation between miR-21-5p and GDF-15 in circulation (p=0.72, p<0.001). We conclude that miR-21-5p possibly has an inductive effect in T2DM patients by causing insulin resistance; also, upregulated GDF-15 expression in circulation hints at the role of TGF-β signalling through miR-21-5p in the pathogenesis of diabetes.

P1261

Regulation of a bacterial RNA repair operon by tRNA fragments

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Most organisms contain repair systems that ligate RNA fragments generated by nuclease cleavage or removal of intervening sequences. A major RNA repair pathway involves RtcB, the ligase that joins pre-tRNA halves after intron excision in animal cells and archaea. In animal cells, RtcB also ligates mRNA encoding the XBP1 transcription factor to initiate the unfolded protein response. RtcB is also present in numerous bacteria where its physiologic roles and substrates are poorly understood. In bacteria, RtcB is often expressed as part of a highly regulated “RNA repair” operon. In this operon, RtcB is encoded adjacent to RtcA, an RNA cyclase that converts 3’-phosphate RNA ends to 2’,3’-cyclic phosphate ends. One role of RtcA may be to generate substrates for RtcB, since RtcB joins RNAs ending in 2’,3’-cyclic phosphate to RNAs containing 5’-OH. The major impediment to studying the functions of this putative RNA repair operon has been a lack of information as to how the operon is regulated. Operon transcription is controlled by RtcR, a sigma54-dependent enhancer binding protein that features an N-terminal ligand-binding domain fused to a C-terminal AAA+ ATPase domain that multimerizes to an active form upon ligand binding. The ligand-binding portion of RtcR contains a divergent CARF (CRISPR-Associated-Rossman-Fold) domain. Canonical CARF domains are bound by cyclic oligoadenylate molecules; however, the ligand that binds RtcR has not been identified. To identify the signals that activate operon expression, we identified mutations that result in transcription of the operon in Salmonella Typhimurium. We discovered that the operon is expressed in the presence of mutations that cause tRNA fragments to accumulate. Some mutations result in DNA damage and we showed that a
novel feature of the DNA damage response in *S. Typhimurium* is the activation of ribonucleases that cleave tRNAs in the anticodon loop, giving rise to tRNA fragments ending in 2',3'-cyclic phosphate. Consistent with the hypothesis that tRNA fragments ending in 2',3'-cyclic phosphate are important for operon activation, overexpression of RtcA increases operon transcription. We showed that the CARF domain binds specific 5' tRNA fragments ending in 2',3'-cyclic phosphate and that RtcR oligomerizes upon binding these ligands, a prerequisite for operon activation. As operon expression is important for *S. Typhimurium* survival following treatment with the DNA damaging agent mitomycin C, our experiments uncover a novel signaling pathway in which DNA damage is coupled via tRNA cleavage to operon activation and implicate the operon in both tRNA and DNA repair.

**P1263**

**Exosomes and microRNAs in maternal milk are important for growth and gut health in neonate mice**  
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**Background:** Human milk contains approximately $2.2 \times 10^{11}$ exosomes per mL, which harbor more than 200 microRNAs. Mammals absorb exosomes and microRNAs from milk, and microRNAs regulate approximately 60% of human genes. Studies in transgenic mice suggest that maternal milk exosomes accumulate primarily in the liver, brain and gastrointestinal (GI) mucosa in neonate pups. **Objective:** Assess whether maternal EVs and their microRNA cargos promote postnatal growth and GI health in neonate mice. **Methods:** Wild-type (WT) pups were fostered to homozygous Tsg101 knockout (KO) dams (impaired exosome biogenesis), heterozygous Dicer KO dams (loss of microRNA biogenesis) or WT dams (control) from synchronized pregnancies (4 pups/dam). We assessed milk exosome counts and microRNA expression, gut development, barrier function, mRNA expression profile in the jejunum, postnatal weight gain, and milk quality and intake. Statistics: unpaired t-test (Tsg101/Dicer vs. control); $P < 0.05$. **Results:** KO of TSG101 and Dicer caused an 80% and 60% decrease of exosomes and microRNAs, respectively, in milk. The loss of milk exosomes and microRNAs led to an up to 20% shorter length of the gut, 20% decrease of villi height and 15% crypt depth, 50% increase in leakiness of the gut (appearance of FITC-dextran in blood), and a 50% loss of postnatal weight gain in pups. Approximately 400 mRNAs were differentially expressed in the jejunums of pups fostered to TSG101 KO dams or WT dams. Nutritional quality of milk and milk intake were not study confounders. **Conclusions:** Mothers communicate with their offspring through exosomes and microRNAs in milk, and the maternal message plays a role in optimal growth and gut health in neonate mice.

**Spindle Assembly 3**

**P1264**

**ZYG-9 and TAC-1 are required for establishing and maintaining acentrosomal spindle poles in *C. elegans* oocyte meiosis**  
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During mitosis, centrosomes act as microtubule organizing centers to create a bipolar spindle, facilitating segregation of DNA into daughter cells. In contrast, oocytes of numerous species utilize an
acentrosomal meiotic spindle to segregate chromosomes and create gametes. Strikingly, it has been shown that the poles of human oocyte spindles are highly unstable, often splitting apart after they form, and spindles with greater instability have more chromosome segregation errors. However, little is known in any system about what stabilizes acentrosomal poles. We have now gained insight into this question using C. elegans as a model. The C. elegans homolog of the microtubule polymerase XMAP215, ZYG-9, has been shown to be concentrated at spindle poles in oocytes and to be required for meiotic spindle assembly. To test for a role in stabilizing acentrosomal poles, we employed the auxin-inducible degron (AID) system to deplete ZYG-9 from either forming meiotic spindles or established metaphase-arrested spindles. When ZYG-9 was depleted prior to spindle assembly, multiple poles formed but these poles could not stably coalesce to establish a bipolar spindle, reminiscent of the pole instability seen in human oocytes. Moreover, acute ZYG-9 depletion from formed bipolar spindles caused severe pole fragmentation, demonstrating that ZYG-9 is required not only to form but also to maintain stable poles. During mitosis, ZYG-9 has been shown to interact with the microtubule-associated protein TAC-1, and ZYG-9 and TAC-1 colocalize interdependently at centrosomes. We found that TAC-1 also colocalized with ZYG-9 at acentrosomal oocyte poles, and like mitosis, TAC-1 and ZYG-9 are interdependent for localization. Additionally, TAC-1-depleted oocytes displayed spindle defects that closely resembled ZYG-9 depletions. Taken together, our data suggests that ZYG-9 is required for both the establishment and maintenance of acentrosomal poles during oocyte meiosis and is likely interacting with TAC-1 to perform this function. Interestingly, previous studies of ZYG-9 in mitotically-dividing embryos found that ZYG-9 localizes to centrosomes but its depletion does not cause pole defects, suggesting that this protein has different requirements at acentrosomal compared to centrosome-containing poles. We used FRAP to analyze ZYG-9 dynamics and found that ZYG-9 is much more dynamic at spindle poles in oocytes compared to at centrosomes in mitosis, supporting the idea that ZYG-9 could function differently in these two contexts. Further investigation of the mechanisms by which ZYG-9 and TAC-1 promote acentrosomal spindle pole stability will be critical for building a more comprehensive picture of how these unique poles compare and contrast to centrosomes.

P1265

Pp2a- B55γ phosphatase regulates proper spindle orientation by orchestrating the cortical levels of dynein adaptor NuMA

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The correct orientation of the mitotic spindle is essential for tissue architecture and organ morphology. Proper spindle orientation further controls accurate distributions of the cell fate determinants during development and in stem cells generation. An evolutionarily conserved cortically anchored protein NuMA localizes dynein, a minus-end directed motor protein complex, at the cell cortex in astral microtubule-dependent manner. There, pulling forces generated by the cortical dynein helps in establishing proper spindle orientation in mitosis. We have shown earlier that NuMA cortical localization is negatively regulated through its phosphorylation at T2055 residue by CdK1/CycB. Thus, only non-phosphorylated species of NuMA at T2055 localizes at the cell cortex. However, the nature of the phosphatase complex that counteracts CdK1/CycB and dephosphorylates T2055 residue remained elusive. In this study, by conducting a candidate-based RNAi screen, we have identified a trimeric PP2A complex consist of B55γ/PPP2CA/PPP2R1B that is responsible for NuMA dephosphorylation at T2055 and proper spindle orientation in mitosis. In vitro reconstitution experiments further reveal that this
complex is sufficient to dephosphorylate NuMA at T2055. We have also shown the relevance of polybasic residues in the vicinity of T2055 for PP2A-B55γ complex mediated dephosphorylation which are essential for spindle elongation. Notably, our data also reveal that phosphorylation and dephosphorylation events at T2055 are reversible. Overall, we have shown PP2A-B55γ counteracts Cdk/CycB activity and spatiotemporally control cortical dynein localization for error-free cell division.

P1266

Chromosomes function as a barrier to mitotic spindle bipolarity in polyploid cells
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Most animal cells are diploid, containing two copies of each chromosome. Establishment of proper bipolar mitotic spindle containing two centrosomes, one at each pole allows accurate chromosome segregation. This is essential for the maintenance of genome stability, tissue and organism homeostasis. However, numerical deviations to the diploid set are observed in healthy tissues. For instance, polyploidy - the doubling of the whole chromosome set - enables the increase of cell size and metabolic rate, and aneuploidy - the gain/loss of whole chromosome - improves stress responses. Strikingly, physiological polyploidy is always associated with low proliferation capacity and levels of aneuploid cells in healthy tissues remain highly debatable. Importantly, whole genome duplication and aneuploidy have been associated with chromosome instability and implicated in tumor initiation and progression. In pathological contexts, polyploidy and aneuploidy arise mainly from mitotic errors. Notably, the consequences of concomitant increase in DNA levels and centrosome numbers on polyploid cells proliferation remains unknown. Using Drosophila melanogaster neural stem cells and human cancer cell lines, we found that in polyploid cell division, an initial centrosome clustering occurs quickly after nuclear envelope breakdown which decreases the number of spindle poles. However, we discovered that the multiple poles are then unable to coalesce together due to the presence of chromosomes in excess. It results in multipolar anaphases. Combining in silico approaches of spindle formation modelling and experimental approaches of laser ablation, we uncovered that chromosomes adopt a spatial configuration comparable to a physical barrier which inhibits spindle pole coalescence. Interestingly, we identified inhibitors of multipolarity. Indeed, an increase in microtubule stability and length rescue bipolar spindle formation in polyploid cells. This study describes how polyploid cells deal with extra-chromosomes and extra-centrosomes and provides a possible link between polyploid and genetic instability. In parallel, in order to obtain a quantitative overview of aneuploid cell levels in healthy tissue, we developed a novel tool to monitor chromosome loss in vivo and at the scale of the whole organism, in Drosophila melanogaster. Our recent unpublished data showed that basal levels of aneuploid cells are very low in most wild type tissues. However, using this tool, we uncovered an unexpected down-regulation of the X chromosome. Interestingly this was specific to neural stem cells of the developing brain. Combination of this aneuploidy probe with long-term live imaging allows us to analyze the mechanisms of aneuploid cells generation and to follow their outcome overtime.
Modeling Reveals Cortical Dynein-Dependent Oscillations in Bipolar Spindle Length

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Proper formation and maintenance of the mitotic spindle is required for faithful cell division. While much work has been done to understand the roles of the key force components of the mitotic spindle, identifying the implications of force perturbations in the spindle remains a challenge. We develop a computational framework accounting for the minimal force requirements of mitotic progression. To reflect early spindle formation, we account for microtubule dynamics and interactions with major force-generating motors, excluding chromosome interactions that dominate later in mitosis. We directly integrate our experimental data to define and validate the model, and then use simulations to analyze individual force components over time and their relationship to spindle dynamics, making it distinct from previously published models. Rather than achieving and maintaining a constant bipolar spindle length, oscillations in pole to pole distance occur that coincide with microtubule binding and force generation by cortical dynein. In the context of high kinesin-14 (HSET) activity, we identify the requirement of high cortical dynein activity for bipolar spindle formation.

Role of centromere in mitotic spindle structure

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Mitotic spindle formation is required for eukaryotic cell division. A mitotic spindle that attaches to all chromosomes via kinetochoore microtubules is needed for accurate chromosome segregation. How cells build the correct size of mitotic spindle to ensure equal segregation of genetic material is not clearly understood. Is the size of the mitotic spindle dependent on the ploidy (genome content) of the cell, or does the cell have a unique counting-based mechanism to “count” the number of centromeres/chromosomes and thereby regulate the size of the mitotic spindle? We have used Saccharomyces cerevisiae as a model system to understand the relation between centromere/chromosome number and mitotic spindle size. Previous studies of yeast of different ploidy suggested that spindle size scaled based on genome content. We extend this work through analysis of genetically modified yeast of fixed genome content but with fewer centromeres/chromosomes. Live cell fluorescence imaging shows that as centromere number decreases, the size of the microtubule organizing center and the number of spindle microtubules also decrease. However, electron tomography reveals unique features of these spindles, suggesting that spindle properties are determined by centromere/chromosome number, chromosome size and centromere organization.
Bud14 in complex with Protein Phosphatase-1 Acts as a Mitotic Exit Inhibitor in Budding Yeast

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Saccharomyces cerevisiae, also known as the budding yeast, orients and elongates its mitotic spindle along its polarity axis in order to segregate one copy of its genomic DNA to the daughter cell. When accurate positioning of the mitotic spindle fails, a surveillance mechanism, named the Spindle Position checkpoint (SPOC), prevents cells from exiting mitosis unless the spindle orientation is corrected. Such mitotic arrest provides cells time to align their spindle correctly before cell division is completed. Mutants with a defective SPOC lose their genomic integrity, become multiploid and aneuploid. Thus, SPOC is a crucial checkpoint for the budding yeast. Yet, a comprehensive understanding of how SPOC mechanism works is missing. In this study, we identified Bud14 as a novel checkpoint protein. We showed that the mitotic exit inhibitory function of Bud14 requires its association with the type 1 protein phosphatase, Glc7. Cells bearing versions of Bud14 that cannot interact with Glc7 or a temperature sensitive mutant of Glc7 were SPOC deficient. Our data indicate that Glc7-Bud14 inhibits mitotic exit by promoting dephosphorylation of Bfa1 during anaphase and limiting the levels of Bfa1 at the spindle pole bodies. Our results support a model in which Glc7-Bud14 works parallel to the SPOC kinase Kin4 in regulating Bfa1, the most downstream effector of SPOC that inhibits mitotic exit.

Intravital imaging and a trainable machine learning-based tool for large-scale analyses of C. elegans germline stem cell mitosis

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Investigating the complex interactions between stem cells and their native environment requires an efficient means to image them in situ. Caenorhabditis elegans germline stem cells (GSCs) are distinctly accessible for intravital imaging and allow the powerful advantages of the C. elegans germ line as a model system for stem cell biology to be merged with cell biological techniques. However, long-term image acquisition and analysis of dividing GSCs can be technically challenging. Here we present a systematic investigation into the technical factors impacting GSC physiology during live imaging and provide an optimized protocol for monitoring GSC mitosis under minimally disruptive conditions. We describe an automated analysis tool, based on tracking and pairing of centrosomes, which allows a variety of mitotic parameters to be rapidly extracted from large-scale datasets, and which is adaptable to other cell types in C. elegans and in other organisms. Using our imaging protocol and analysis method, we investigate spindle orientation in GSCs relative to the niche and the distribution of mitoses within the stem cell pool. In sum, we provide technical and analytical tools that open the gates for large-scale screening studies of multiple mitotic processes in GSCs dividing in situ, in an intact tissue, in a living animal, under seemingly physiological conditions.
EB1 photoinactivation reveals distinct roles of microtubule plus end dynamics in spindle size and orientation.

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Equal distribution of the genetic material during cell division is accomplished by the mitotic spindle, a highly dynamic molecular machine comprised of different microtubule populations that connect to chromosomes, the cell cortex, or other spindle microtubules. Spindle size, position and orientation within metaphase cells is critical for correct chromosome segregation especially in the context of asymmetric cell divisions in development and differentiation. The microtubule end-binding protein EB1 is abundant on the ends of growing microtubule plus ends in metaphase cells and mediates interactions with a network of so-called +TIPs at growing microtubule ends. Yet, how EB1 and associated +TIPs contribute to spindle dynamics and function is incompletely understood. π-EB1 is a photo-inactivated EB1 variant that results in rapid and reversible dissociation of the +TIP network from growing microtubule ends when exposed to blue light (van Haren et al., Nat. Cell Biol. 20:252-261). Here we use π-EB1 photoinactivation in cells in which endogenous EB1 was deleted by CRISPR/Cas9 genome editing to ask how EB1 and associated +TIPs contribute to spindle dynamics and function with high spatial and temporal accuracy. Global π-EB1 inactivation throughout whole mitotic cells resulted in a ~25% shortening of metaphase spindles within minutes that was fully reversible when blue light exposure was switched off. Spindle shortening was not a result of photodamage and did not occur in control cells in identical experimental conditions. Experiments in which blue light exposure and thus π-EB1 inactivation was restricted to either cortical or central parts of the spindle evoked similar, but smaller spindle shortening responses. Similarly, half spindle illumination also caused spindle shortening and was accompanied by spindle pole movement away from the cell cortex only in the light-exposed half. Together, these data indicate that steady state spindle shape relies on a balance of cortical pulling and central pushing forces that depend on EB1-mediated interactions with dynamic microtubule ends. Strikingly, and in stark contrast to interphase-mediated interactions, the microtubule growth rate in mitosis was not affected by π-EB1 inactivation, indicating that spindle shortening is not simply a result of microtubule growth inhibition. Lastly, when we inactivated π-EB1 asymmetrically at cortical regions near both spindle poles, the spindle axis rotated away from the blue light exposure axis indicating that EB1-mediated interactions are required to properly engage cortical dynein-mediated pulling on astral microtubules.

Therapeutic Approaches

Novel Thienopyrazole Promotes Potent and Selective Apoptosis in Human Cancers

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In this study, a thienopyrazole derivative with cancer-selective cytotoxicity was identified in a high-throughput chemical library screening to identify novel therapeutic candidates. After 48 hours of
exposure, this compound demonstrated potent and consistent cytotoxicity against a panel of human cancer cell lines at nanomolar to low micromolar concentrations. Mechanistic analyses subsequently revealed dose-dependent apoptotic cell death, interference with cell cycle progression, and inhibition of microtubule polymerization. These findings merit the continued development of this compound as a therapeutic agent in the treatment of human cancers, particularly those of hematopoietic and cervical origin.

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Multiple Mechanisms of Synergy Result from Combined Targeting of Tropomyosin and Microtubules in Ovarian Cancer Cells

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Targeting the tropomyosin 3.1 (Tpm3.1) -containing actin-cytoskeleton using anti-tropomyosin compounds (ATM) potentiates the anti-cancer efficacy of both microtubule depolymerising and stabilising agents. Previous findings suggested that ATMs synergise with microtubule depolymerising agents by enhancing mitotic defects. In this study, we have determined the mechanism of synergy of ATMs with both a microtubule depolymeriser and a stabiliser using an ovarian cancer cell line, OVCAR4, as the study model. The antiproliferative and cytotoxic efficacy of an ATM and vinorelbine (VNB) or paclitaxel (PTX) were evaluated using the MTS cell viability assay and an Annexin V apoptosis assay, respectively. Live cell imaging was used for cell cycle and cell fate analysis. The localisation of BubR1 on kinetochores was examined using immunofluorescence to measure the activity of the spindle assembly checkpoint (the SAC). Expression of key proteins involved in interphase progression were analysed using Western blots. We found that ATM synergises with either VNB or PTX in the reduction of cell viability, but only synergises with VNB in the induction of apoptotic cell death. ATM significantly prolongs VNB induced mitotic arrest with increased activity of the SAC, resulting in almost one third of all cells dying in mitosis. In contrast, cotreatment of ATM and PTX did not shift the mitotic cell fate caused by PTX alone, however, they result in a subsequent arrest in G1 phase. Surprisingly, cells exposed to the combination of ATM and VNB that ultimately undergo mitosis, arrest in the subsequent G1. Both drug combinations result in decreased cyclin D1 and E1 compared to the single agent treatments alone. We find significant upregulation of p21Cip and p27Kip, but not p16INK4a associated with both drug combinations and consistent with the interphase arrest. We conclude that while ATMs synergies with anti-microtubule drugs, the mechanism of synergy between ATMs and microtubule depolymerisers differs from ATMs and microtubule stabilisers, at least in mitosis. This in turn suggests that Tpm3.1-containing actin filaments are involved in multiple functions of the microtubule network during a cell cycle.

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Two modes of cargo delivery by cell penetrating peptides: membrane permeation and lysis

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Cell penetrating peptides (CPP) containing positively charged amino acids such as arginines are powerful agents for delivery of proteins or nucleic acid cargoes for therapeutic, diagnostic or gene editing applications. The CPP nonaarginine (R9), for example, can deliver the Cas9 enzyme and sgRNA in the CRISPR/Cas9 gene editing technique. Membrane permeabilities of such bulky cargoes are greatly increased when covalently or physically complexed to CPPs. However, the mechanisms of translocation are not understood. Here we developed a first principles quantitative model of polycation-membrane interactions that identified two modes of cargo delivery mediated by R9 and other CPPs, with distinct delivery kinetics. The operative mode depends on target compartment size. The model calculates membrane adhesion, evolution of hemifused complexes and transitions to fusion (Warner and O'Shaughnessy, 2012), adapting a kinetic framework for tension-generated membrane lysis via pore nucleation and growth (Evans et al., 2003). We find negatively charged phospholipid membrane-enclosed compartments (e.g. cells or liposomes) are strongly adhered and contracted by polycations, boosting their tension and catalyzing their hemifusion in a polycation concentration-dependent manner. Three outcomes are possible, with vesicle size-dependent relative weighting. (1) Membrane fusion. Rupture of the high tension hemifusion diaphragm (HD) by nucleation and growth of a membrane pore fuses the adhered compartments. (2) Vesicle Lysis. This occurs if the vesicle membranes rupture faster than the HD. (3) Dead end hemifusion. These predictions agree quantitatively with experiments studying fusion of giant unilamellar vesicles (GUVs) mediated by Ca and Mg cations, used for decades as a protein-free model of biological fusion (Nikolaus et al., 2009). All 3 outcomes were observed experimentally. We applied our model to R9 acting on 10 µm diameter GUVs, and on ~ 400 nm diameter LUVs, as in the experiments of (Alloio et al., 2018). At the concentrations employed, we find R9 ruptures the GUVs after ~ 2 mins, but LUV rupture is unobservably slow (theoretical rupture time ~ 1000 mins). This agrees with experiment: GUVs ruptured rapidly, but LUVs remained intact and R9 increased their membrane permeability ~ 10,000-fold (Shimanouchi et al., 2009), with a dye contents release time of ~ 50s. Thus, we identify two modes of R9-mediated cargo delivery. (1) For smaller ~ 100 nm -sized compartments, the increased membrane permeability allows non-destructive R9-mediated passage of cargo, possibly accompanied by R9. (2) For cell-sized compartments, R9 achieves fast cargo entry by membrane lysis, which cells can likely repair, long before full contents exchange by non-destructive membrane permeabilization.

P1275

Optimizing production and purification of bacterial outer membrane vesicles
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Outer membrane vesicles (OMVs) produced by Gram-negative bacteria, such as Nontypeable Haemophilus influenzae (NTHi), are spherical, membrane-enclosed entities that contain periplasmic and outer membrane proteins, as well as other cellular contents. Bacteria often produce OMVs under stressful environmental conditions as a defense mechanism and to remove misfolded outer membrane proteins that could compromise the integrity of the bacterial cell. Due to their potentially rich antigenic content, OMVs are a relatively new target in the field of vaccinology. The objective of this work is to optimize the yield of purified NTHi OMVs with the overarching goal of using OMVs in a vaccine formulation to protect against NTHi infection. We employed several strategies to increase the yield and
purity of OMVs, including the use of ion exchange column chromatography and ultracentrifugation. To quantify and analyze the OMV samples, we used a standard Bradford assay, gel electrophoresis, immunoblotting, nanoparticle tracking analysis (NTA), and transmission electron microscopy (TEM). Preliminary results suggest that ion exchange chromatography allows for rigorous purification, but greatly lowers the overall yield of OMVs. Based on this work, we propose the need for a better isolation method that allows for more efficient purification of the OMVs, ideally into subpopulations based on size.

P1276

**Controlling intracellular transport to tune cell-based (immuno)-therapies**

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Recent years have brought a major change in the paradigm of tumor treatment. In addition to the collection of classical treatments (surgery, chemotherapy, radiation), the use of immunology-based approaches has opened new roads with promising results for cancer treatment. In particular, cell-based therapies based on the expression of a Chimeric Antigen Receptor (CAR) at the surface of T cells (CAR-T) that mimics T cell receptor activity have shown impressive effects to cure certain B cell malignancies. Two products, Kymriah and Yescarta, were approved by the U.S. Food and Drug Administration (FDA) and by European Medicines Agency (EMA) starting from 2018. Although very efficient, these treatments come with critical side effects, that can be life threatening. These includes cytokine release syndrome and neurotoxicities, that are correlated with an overactivation and expansion of the CAR-T cells. In addition, only limited success has been obtained against solid tumors or against non-immunogenic tumors for example. Because these cell-based therapies rely on efficient protein trafficking, we decided to exploit our knowledge in intracellular transport control to try to improve cell-based therapy and in particular CAR-T cell. More generally, we strongly believe that the power of cell biology can be harnessed to develop novel tunable cell-based therapies. Several years ago, we have set-up a system (the Retention using selective hooks, or RUSH, system), which is now widely used, that allows to synchronize the transport of virtually any secretory protein cargos. Cargos bearing a Streptavidin-binding peptide (SBP) are retained in the endoplasmic reticulum (ER) using a stably located hooking protein fused to Streptavidin. Addition of biotin allows rapid release of the cargo and its export to its target compartment. We modified our system so that the transport and secretion of CAR-T proteins, or modulating factors like certain cytokines or chemokines, can be controlled by the simple addition of biotin in living cells, and in particular in T cells. With this system, that we call CELLTune, we could elicit interferon or IL2 signaling for example. We could also reprogram T cell using such a modulated CAR-T so that toxicity can be controlled by biotin. We are now bringing the system in vivo. Our first results indicate that we can control the secretion of cytokines from reprogrammed and grafted cells in vivo by injecting mice with biotin. We could also stimulate release by simple feeding. We are now carrying out experiments to validate the use of our tuned system (CELLTune) to control tumor eradication in vivo. Bringing such a tunable system in the clinic may be a game changer in many situations and we are now implementing a large set of regulatory factors in our system to broaden its use.
Oral eggshell membrane administration attenuated lung fibrosis via type III collagen- and decorin-mediated wound healing

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Chicken eggshell membrane (ESM) is a natural wound healing composite material. ESM is mainly composed of cross-linked fibrous collagens that act on the skin, and also have various ameliorative effects such as osteoarthritis of the knee, joint and connective tissue when ingested and absorbed as a supplement. We previously showed that ESM affects dermal fibroblasts expression of COL3 and DCN, and contributes to skin resilience. ESM might affect or improve lung and skin function. Nevertheless, ESM has a physiological effect in vivo after digestion, absorption, and metabolism; however, the detailed cellular mechanism of ESM remains unclear. Therefore, in this study, we successfully obtained tritium (³H)-labeled ESM by the Li⁶(n, α)³H reaction by neutron irradiation, and then examined the transfer of the labeled ESM hydrolysate into the bloodstream after digestion and absorption. In addition, we also examined whether oral ESM supplementation improves lung and skin function in human and the BLM-induced pulmonary fibrosis model served as an internal wound healing model, and the wound healing effects of ESM were tested using COL3 and DCN as key markers, focusing on the extracellular matrix. The results of gastric administration of ³H-labeled ESM powder in mice showed that all examined organs contained ³H-labeled products. Oral 14-28 days ESM intake elevated COL3 and DCN in mouse lung and skin tissues. A significant increase in skin elasticity and lung FEV1/FVC was observed 8 weeks after the double-blind ESM supplementation to 30 adults. In addition, DCN, a regulator of many growth factors, is a PG that directs TGF-β in the direction of health maintenance and is particularly essential in pulmonary fibrosis. We tested the anti-fibrotic effect of ESM in a pulmonary fibrosis model conveniently created by single dose administration of bleomycin (BLM) into the trachea, and found that a significant increase in COL3 in the bronchioles and lung on the 1st and 3rd day and DCN levels correlated with better healing after 2 weeks. Lung fibroblast on ESM peptides expressed significantly high COL3 and DCN levels, and suppressed the nuclear translocation of SMAD by TGF-β1 stimulation. The improved correlation between FEV1 / FVC and arm skin elasticity with ESM supplementation indicates that the effects of ESM are systemic. In conclusion, aging-dependent lung and skin fibrosis might be prevented by ESM intake, which favors COL3- and DCN-mediated wound healing.

Inhibition of Renin Angiotensin System with Losartan Improves Glucose Metabolism and Ameliorates Insulin Resistance in Prediabetic Rhesus Monkeys (Macaca mulatta)

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Prediabetes is an asymptomatic condition defined as impaired fasting glucose levels and/or glucose tolerance, where glucose levels are not high enough to be diagnosed as diabetes. This condition is an important risk factor for type 2 diabetes (T2D), stroke and cardiovascular disease. Angiotensin II (Ang II) can induce insulin resistance (IR) and T2D by altering insulin sensitivity (IS) and glucose uptake. This suggests that activation of the renin-angiotensin system (RAS) may play a role in the onset of prediabetes. Whether inhibition of RAS with losartan, an Ang II type 1 receptor blocker, improves IS and glucose homeostasis in prediabetic rhesus monkeys has not been explored. We hypothesize that losartan will ameliorate IR by improving metabolic health in prediabetic monkeys. Our objective is to characterize a cohort of prediabetic rhesus monkeys to examine the effects of losartan on metabolic parameters associated with prediabetes. We identified prediabetic middle-age monkeys by measuring plasma levels of fasting glucose (FG) and insulin. Prediabetic monkeys were treated with losartan (30mg/day) and the plasma samples were obtained at 0 (baseline), 3, 6, and 12 months using standard protocols. Healthy untreated middle-age rhesus monkeys were also analyzed as a control group. We measured FG and insulin, and glucose and insulin responses after a glucose challenge by the intravenous glucose tolerance test. IS was measured by the quantitative insulin sensitivity check index. Prediabetic monkeys showed a significantly higher FG (p=0.001), compensatory higher insulin levels, and significantly higher IR (p=0.03) when compared to healthy monkeys. Losartan treatment improved glucose homeostasis, insulin levels, and IS in prediabetic monkeys. Interestingly, glucose levels at 1 minute after the glucose challenge significantly decrease (p=0.02) in prediabetic monkeys after losartan when compared to baseline levels of the same group. However, losartan had no significant changes in the mean area under the curve for glucose and insulin after a glucose challenge. Our results suggest that losartan improves glucose metabolism and ameliorates IR in prediabetic monkeys. Therefore, inhibition of Ang II action might be an effective pharmacological approach for the treatment of IR and the prevention of T2D. Supported by R36-AG065725-01 (JLD), P40RR003640, U54-MD007600.

Trafficking in Neurons

P1279

**Schizophrenia-linked protein tSNARe1 regulates endolysosomal trafficking in cortical neurons.**


Schizophrenia is a severe and heritable neuropsychiatric disorder; the etiology of which is the result of genetic variation in many genes. Genome-wide association studies (GWAS) identified 145 genome wide significant (GWS) loci, implicating more than 300 candidate genes as potential risk factors for schizophrenia. However, the impact of these genes in schizophrenia pathogenesis has not been studied. The next critical step is to investigate the function of these genes and how they might be dysregulated in schizophrenia. **T5NARE1**, which encodes the protein tSNARe1, is one of the high confidence candidate genes, but the cellular or physiological function of tSNARe1 is presently unknown. Here we define the major gene products of **T5NARE1** in the human brain and their cytoplasmic localization and function in the endolysosomal system of cortical neurons. We identified four isoforms of tSNARe1 in human brain, all of which contain a syntaxin-like Qa SNARE domain. SNARE proteins are involved in the fusion of opposing lipid bilayers, and the transmembrane domain of the Qa SNARE is thought to be critical for membrane fusion. However, RNA-sequencing data from adult and fetal human brain suggest that the
most abundant isoforms of tSNARE1 lack a transmembrane domain. This suggests an exciting hypothesis in which brain tSNARE1 acts as an inhibitory SNARE (i-SNARE) to negatively regulate membrane trafficking events. We find that brain tSNARE1 isoforms localize to compartments of the endolysosomal network. The non-transmembrane domain containing isoforms, including the most abundant isoform tSNARE1c, most frequently populate compartments of the late endosome. Live-cell three color trafficking assays suggest that tSNARE1 regulates trafficking between the late endosome and the lysosome in the developing neuron, and ongoing experiments will determine if tSNARE1 modulates rates of clathrin-mediated endocytosis. Determining which trafficking events tSNARE1 regulates is critical towards understanding how its dysfunction contributes to schizophrenia pathogenesis.

P1280

**Neonatal lethality and impaired axonal lysosome transport in mice with KO of the myrlysin (BORCS5) subunit of BORC**

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The ability of lysosomes to move throughout the cytoplasm is critical for various cellular functions such as autophagy, cell migration and tumor invasion. This ability is particularly crucial in neurons because of their extreme asymmetry and length of axons and dendrites. We recently discovered an eight-subunit complex named BORC that mediates sequential recruitment of the small GTPase ARL8, the adaptor protein SKIP, and kinesins-1 and -3 to lysosomes, promoting their movement toward the plus-end of microtubules in the periphery of non-polarized cells. Knock-out (KO) of any of the BORC subunits causes clustering of lysosomes in the pericentrosomal area of the cell. A recent study revealed the presence of a single-nucleotide, splice-acceptor variant of the BORCS5 gene, encoding the myrlysin (BORCS5) subunit of BORC, in a patient with global developmental delay, corpus callosum agenesis, seizures, polymicrogyria and abnormality of the cerebral cortex. Furthermore, single nucleotide polymorphisms causing altered expression of the diaskedin (BORCS7) subunit of BORC have been associated with schizophrenia, emphasizing the importance of BORC in neurons. To analyze the requirement of BORC for neuronal function, we used the CRISPR-Cas9 system to generate a myrlysin-KO mouse. We found that the resulting KO embryos grew to term, but the pups died of suffocation immediately after birth. To address the importance of BORC in neuronal organelle transport, we prepared cultures of hippocampal neurons from control and myrlysin-KO embryos. The neurons were transfected with plasmids encoding lysosomal and synaptic vesicle markers fused to fluorescent proteins. We found that myrlysin KO prevented transport of lysosomes into the axon but not the dendrites. In contrast, there was no effect in the transport of synaptic vesicle precursors (SVPs) into the axon. Analysis of brain tissues and neuromuscular junctions from WT and KO embryos confirmed that BORC is necessary for lysosomes, but not for SVPs, to enter the axon and to reach synaptic terminals. These findings indicate that in mouse neurons BORC plays a role in the transport of lysosomes but not SVPs into the axon. Neuropathogenesis caused by mutations in the BORC complex could thus result from impaired lysosome transport into the axon.
P1281

Development of a LYST-deficient glutamatergic neuronal model of Chediak-Higashi Syndrome

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Neurons are highly polarized cells due to the microtubule orientation in their axons that serves as tracks for the intracellular transport of organelles and macromolecular complexes. The lysosomal distribution, mobility, size and number are tightly regulated to maintain cellular homeostasis. Under pathological condition, such as Chediak-Higashi Syndrome (CHS), dysregulation of the lysosomal pathways occurs. CHS is a rare, lysosome-related organelle disorder associated with progressive neurological dysfunction, a bleeding diathesis, high susceptibility to infections and albinism. It is caused by bi-allelic mutations in the lysosomal trafficking regulator (LYST) gene that encodes a 429 kDa protein. To date, several cell and animal models of CHS have been investigated, but none of them consistently recapitulates the neurological phenotype seen in patients. Despite its name and presumed function, there is no clear evidence supporting the role of LYST in regulating lysosomal trafficking. In this study, we investigate the functions of LYST in lysosomal regulation using a neuronal cell model applying CRISPR/Cas-9 technology to knock-out LYST in induced pluripotent stem cell (iPSC). These cells have an inducible Neurogenin 2 expression cassette integrated into the AAVS1 safe-harbor locus, enabling the production of glutamatergic neurons. The perinuclear degradative lysosomes of LYST-deficient glutamatergic neurons are larger in size but fewer in number, indicating that LYST regulates lysosomal biogenesis. To further explore the dynamics of lysosomes in these neurons, we are using live cell imaging techniques to characterize the role of LYST in the lysosomal fusion/fission events and trafficking. This model provides the first evidence that LYST-deficient glutamatergic neurons have lysosomal abnormalities and will help us in understanding the neurological phenotype associated with CHS patients.

P1282

Huntingtin and Rab7 comigrate on a retrogradely moving axonal endolysosome

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Huntington’s disease (HD), is a neurodegenerative disease hallmarked by an expansion of polyglutamine (PolyQ) in Huntingtin (HTT). Although HTT has been shown to move bi-directionally within axons and loss of HTT was shown to cause axonal transport defects, the type of cargo carried by HTT-containing vesicles remain unclear. Previously, we found that HTT mediates the retrograde movement of Rab7-containing axonal compartments and that HTT and Rab7 comigrate within axons. In this study, we used Drosophila genetics to unravel a unique axonal endolysosome containing both HTT and Rab7. Complementing our previous findings, we observed a disruption in the retrograde population of Rab7-containing axonal compartments in the context of HTT deficiency. Moreover, a genetic reduction of Rab7 caused a significant perturbation to the retrogradely moving population of HTT-containing axonal compartments. Interestingly, we observed that HTT-Rab7 axonal compartments display a retrograde movement bias, which were found to be likely aided by RILP or HIP1, in addition to molecular motors.
kinesin-1 and dynein. Although we found HTT co-migrating with ATG8-containing axonal compartments, ATG8 could label multiple different compartment types; therefore, we looked at ATG5, which plays a role in autophagosome assembly, and SYX17, which plays a role in endolysosome-autophagosome fusion. Interestingly, we found that the axonal movement of HTT-Rab7 compartments likely becomes disrupted with genetic reductions in SYX17, but not ATG5. Remarkably, pharmacological disruption of endolysosomes via Chloroquine or Bafilomycin-A1 disrupted the axonal movement of HTT-Rab7 compartments, but synaptic vesicles continued to move robustly. Moreover, we found that HTT-Rab7 axonal compartments also co-contain LAMP1 or PI(3)P phospholipids, which all accumulated in axons together with Chloroquine-induced endolysosome disruption, further defining a unique HTT-containing endolysosome in axons. Supporting our findings in Drosophila, we found that HTT and Rab7 associate together on light membrane fractions isolated from mouse brain tissue, which also contained molecular motors and components of endolysosomes. In the context of pathogenic HTT with polyQ expansion, we found that axonal accumulations of HTT-Q138 sequester Rab7 and LAMP1, but not ATG5. Together, these data strongly suggest that HTT and Rab7 co-migrate on a retrogradely moving axonal endolysosome, which likely becomes disrupted with polyQ-HTT.

Tumor Microenvironment: Cytoskeleton and the Extracellular Environment

P1283

Microtubule dependent nuclear translocation of STAT3 in M2 macrophages: Implications for tubulin targeted chemotherapeutics

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Microtubules are essential components of cell cytoskeletal system involved in important cellular functions. Microtubule targeting agents are a conventional and widely used approach in chemotherapies that target the rapidly dividing tumor cells by blocking mitosis. However, their effect on tumor microenvironment and especially on terminally differentiated cells such as Tumor-associated macrophages (TAMs) is still not known. Tumor-associated macrophages (TAMs) mainly comprises of pro-tumoral M2 phenotypic macrophages that assist in tumor progression. Therapeutic approaches to mitigate the pro-tumoral properties of the M2 macrophages are an attractive strategy to modulate tumor microenvironment. In this work, we investigated the role of microtubules in functioning of M2 macrophages. To mimic TAMs in an in-vitro system, THP-1 monocytes were differentiated into M0 macrophages and further polarized into M2 macrophages using 20 ng/ml of IL-4 and IL-13 each. The Conditioned Medium (CM) of M2 macrophages was collected and its effect on metastatic potential of breast cancer cell lines, MCF-7 and MDA-MB-231 was assessed. To decipher the role of microtubules in the functioning of TAMs, their microtubules were depolymerized using 2-Methoxyestradiol, a microtubule depolymerization agent. The concentrations of 2-Methoxyestradiol which did not induce cell death but depolymerize TAM microtubules was used. We found that the CM of the macrophages with depolymerized microtubules could not induce cell migration, epithelial-mesenchymal transition or MMP secretion as efficiently as in case of control macrophages. The data suggested that disruption of the microtubule network of M2 macrophages compromised their metastasis promoting properties. Our data further suggests that microtubule depolymerization in M2 macrophages prevents the nuclear translocation of STAT3, the transcription factor that multitasks in promoting pro-tumoral characteristics...
of M2 macrophages by increasing the secretion of anti-inflammatory cytokines. This results in reduced expression of metastasis promoting anti-inflammatory cytokines such as IL-10, CCL18, TGF-β and hence prevents the TAMs to propel metastasis in breast cancer cells. Our data suggests that STAT-3 nuclear translocation is microtubule dependent and may contribute to anti-tumoral effects of microtubule targeting chemotherapies. **Keywords:** Microtubules, Tumor Associated Macrophages, Breast cancer, metastasis, MMPs, STAT3

P1284

**Actomyosin-Dependent Force Generation is Hijacked to Promote Tumor Progression via ECM Remodeling and Immunosuppression**  
**Y. Chen, W. Jung, S. Park, J. Chen; Johns Hopkins University, Baltimore, MD.**

It has been observed that many types of cancer cells, including breast cancer, lung cancer, prostate cancer and melanoma, can generate higher forces via actomyosin contraction, compared to their normal counterparts. It is known that higher actomyosin forces generated by cancer cells power cancer cell migration to invade distant tissues, driving further cancer development towards metastasis. We recently found that in addition to increase cell motility, cancer cells exploit the elevated actomyosin force generation to remodel extracellular matrix (ECM) and to deplete important co-stimulatory receptors on antigen presenting cells (APCs), facilitating tumor progression. In an in vitro organoid system, we observed that in a force-dependent manner, breast cancer cells align ECM fibrils and thereby enhance the diffusion of exosomes containing cytokines activating stromal cells to exhibit cancer-associated fibroblast (CAF) phenotypes. Suppression of force generation by perturbing Rho signaling, or ECM remodeling abolishes the enhancement of exosome diffusion and the subsequent CAF induction. Moreover, we also observed that after cell-cell contact was established between breast cancer cells and antigen presenting cells, CTLA-4, a key molecule in immunosuppression, expressed on the breast cancer cells bind to CD80 expressed on the APCs, and underwent trans-endocytosis to deplete CD80. CD80 is a costimulatory receptor promoting T cell activation. Force measurement and live cell imaging revealed that upon binding to CD80, forces generated by breast cancer cells and transmitted via CTLA-4 are sufficiently strong to displace CD80 from the surface of APCs to be internalized by breast cancer cells, significantly attenuating the capacity of APCs to activate T cells. In contrast, normal mammary epithelial cells do not generate sufficient forces to internalize CD80. Inhibiting force generation by small molecules in cancer cells increases T cell activating capacity of APCs. Taken together, our results suggest targeting actomyosin force generation specifically in cancer cells can effectively stall tumor progression, providing a new strategy to treat cancer types where cancer cells generate abnormally high forces.

P1285

**Pre-metastatic stiffness induces hMSC differentiation into CAFs**  
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MSCs are known to home into the tumor microenvironment and differentiate into cancer-associated fibroblasts (CAFs). While stiffness-dependent differentiation of MSCs into different lineages is well appreciated, whether or not stiffness which is altered in cancers also influences differentiation into CAFs remains unexplored. Here we have addressed this question by culturing human MSCs (hMSCs) on PA-
gels of varying stiffness (0.5 kPa, 2 kPa, and 5 kPa) in the presence and absence of conditioned media (CM) from breast cancer cells (MDA-MB-231). Our results suggest that 2 kPa substrates, which correspond to the bulk stiffness of pre-metastatic tumor microenvironment, leads to increased proliferation, migration, and differentiation of hMSCs into CAFs at 1-week time point. Interestingly, after this initial conditioning, hMSCs continue to express the CAF marker α-smooth muscle actin (α-SMA) even after another week in culture in the absence of CM, indicative of stable differentiation in a TGF-β/contractility dependent manner. Together, our results suggest that pre-metastatic environments optimally induce CAF differentiation in hMSCs.

P1286

**Mapping cellular interactions between cancer and normal cells via synthetic Notch ligand/receptor pairs**


Understanding how cancer cells interact within its surroundings is critical for elucidating fundamental mechanisms of cancer biology and metastasis. We utilized the synthetic Notch ligand/receptor pair system (synNotch - Morsut L., Roybal KT., et al. Cell 2016) to track the physical interactions between normal and cancer cells with an ultimate goal of investigating the changes in gene expression profile of normal cells before and after the interactions occur. Here we use RPE1 (immortalized human retinal pigment epithelial) normal/non-cancer, and ACC01 (immortalized patient derived adenoid cystic carcinoma) cancer cells, expressing synthetic Notch ligand/receptor pairs to measure the dynamics of touch-based interactions between normal-normal, cancer-cancer and normal-cancer cells. We find that, when co-cultured with ligand-presenting cancer cells, syn-Notch receptor carrying normal cells (1) can internalize the ligand molecules (trans-endocytosis) on average ~500 (+/- 280) times more when compared to receptor negative cells, (2) have detectable ligand molecules in their cytoplasm up to 24 hours post termination of interactions, (3) can be programmed for activation/inactivation of fluorescent reporters for longer term tracking. Moreover, by performing RNA-seq analysis on fluorescently sorted receptor positive normal cells, we compare the gene expression profiles of co-cultured cells with or without trans-endocytosis. This differential analysis provides a basis for a physical interaction dependent gene signature in the non-cancer/cancer cell microenvironment. Ultimately, we seek to integrate our system into mice to study how an individual cancer cell metastasizes and identify all of the non-tumor host cells with which it comes into contact during the metastatic process. We envision the same sensor mice will be valuable model to study other processes, such as neurogenesis or development of the immune system.

P1287

**A type III collagen-rich extracellular matrix niche regulates tumor dormancy**

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Background. Metastasis is the primary cause of death in cancer patients. However, metastasis occurs years after primary tumor removal. This delay is a consequence of tumor dormancy, a reversible growth arrest that can be regulated by the interaction of disseminated tumor cells with the microenvironment. Extracellular matrix (ECM) is a key component of the microenvironment that provides signals to the tumor cells and regulates tumor progression. Here, we investigate the role of tumor-derived ECM and tumor cell/ECM interactions in dormancy. Methods. We used dormancy models of human head and neck squamous cell carcinoma as well as murine mammary carcinoma coupled with multiphoton intravital imaging to define how assembly of ECM by dormant cells sustain their quiescence. Results. SHG imaging showed that ECM of dormant cells is mainly formed of a curly ECM whereas proliferative tumors set up collagen fibers straight. Transcriptome analysis of dormant and proliferative tumors revealed that dormant ECMs are highly enriched in collagens. Interestingly, depleting COL3A1 in dormant cells results in their awakening and restoration of tumor growth in vivo. We identified DDR1, a collagen receptor for these collagens, as upregulated in dormant cells upon dormancy onset (i.e TGFβ2). Col III-rich microenvironments induce DDR1 expression and the entrance in dormancy. DDR1 downregulation leads to the reactivation of dormant cells in vivo. ECM-proteomic analysis revealed that DDR1 is required to sustain tumor cell-derived COL3A1 expression in dormant cell, ECMs suggesting that DDR1 sustain dormancy by regulating the assembly of a pro-dormant ECM enriched in COL3. Moreover, we further identify a transcription factor network activated downstream DDR1, involving STAT1, that contribute to dormancy of cancer cells and to the assembly of this tumor self-made pro-quiescence ECM. Conclusion. We demonstrated that upregulation of DDR1 prime cancer cells to secrete and assemble a pro-quiescence ECM through STAT1 signaling.

P1288

In depth proteome profiling of stage specific colon cancer cell secretome

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Despite major improvements in knowledge, therapies and clinical management, cancer generally remains a deadly disease. Globally colorectal cancer (CRC) is the third most common cancer in women and the fourth most common cancer in men. Cancer cells acquire several capabilities to fully develop their malignant phenotypes. For many of these acquired abilities, arrays of tumor cell secretory factors are necessary to initiate and maintain these processes. These factors are collectively referred to as the ‘cancer secretome’, which includes all proteins secreted, shed or leaking from a cancer cell or tissue under certain conditions and at a certain time. The cancer secretome can thus be considered to represent the tumor microenvironment that plays a key role in tumor-promoting processes, such as angiogenesis, migration, and invasion. Here using a Mass spectrometry approach to characterize the secretome of different subtypes of CRC cell lines, we have identified 1507 and 2466 proteins from the HT29 and DLD1 cellular secretome, respectively. SignalP analysis disclosed 113 (HT29) and 263 proteins (DLD1) with a signal peptide that were secreted via the classical pathway; SecretomeP analysis predicted
a total of 397 (HT29) and 709 (DLD1) proteins that could be secreted via the non-classical pathway. Gene Ontology (GO) analysis revealed that most of the proteins were related to Protein Metabolism, Signal Transduction, and Cell Communication. In using SWATH-MS quantitative analysis, 121 proteins were down-regulated and 7 were up-regulated in the HT29 secretome, in comparison with DLD1. Further, pathway analysis revealed that most of the identified proteins were associated with the platelet-derived growth factor receptor (PDGFR) and the epithelial to mesenchymal transition (EMT) signaling pathways. In conclusion, this study characterizes the secretome signature of the HT29 (Stage 2) and DLD1 (Stage 3) CRC cell lines, providing a list of putative CRC biomarkers related to a specific tumor stage.

P1289

The Microbiome Mediates Carcinogenic Alterations of the Mammary Gland in the Context of Obesity

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Several mechanisms have been shown to drive breast cancer progression in obesity, however, if and how obesity contributes to breast cancer initiation is poorly understood. Obesity alters adipose tissues, the immune environment, and the microbiome in ways that could potentially increase breast cancer risk. Our preliminary experiments with a mouse mammary carcinogenesis model indicate that diet-induced obesity alters the microbiome in the gut and the mammary gland and leads to decreased tumor-free survival and tumor latency, and increased tumor weights and multiplicity. Microbiome metabolites such as the toll-like receptor-agonist, lipopolysaccharide (LPS), could directly affect breast epithelial cells. These metabolites were shown to be elevated in the plasma of obese mice. Preliminary experiments in a 3D culture model of breast glandular units (acini) show that LPS disrupts tight junctions (TJ), which strictly define apical polarity. The loss of apical polarity is a known functional biomarker of breast cancer risk. Other biomarkers of risk include DNA damage and reactive oxygen species (ROS) generation. Our preliminary data also indicate elevated ROS in breast acini treated with LPS. A recent finding by our lab hinted to the importance of redox balance in polarity maintenance, and hence, ROS generation by LPS could provide a mechanism for polarity loss. The outcomes of our study underscores the importance of this almost-forgotten organ, the microbiome, and the need to consider in prevention and treatment strategies in the future. In conclusion, we show that obesity-modulated gut microbiome increases breast cancer risk, at least partly, through the involvement of microbiome metabolites such as LPS.
Wednesday, December 16, 2020, 1:00 pm

Actin and ABPs in Cellular Dynamics

P1290

Resolution of nuclear versus cytoplasmic activities of the chromatocytoskeletal remodeler SETD2
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Initially identified in 1998 as Huntingtin Yeast Partner B, HYPB is now known as the histone methyltransferase SETD2 that ‘writes’ the H3K36me3 methyl mark on chromatin. Although SETD2 is well characterized as a chromatin remodeler, for over two decades the nature of the SETD2 interaction with Huntington (HTT) protein has remained a mystery. Thus, the lack of data on this interaction remains a blind spot in our understanding of HTT function, as well as the pathogenesis of diseases linked to SETD2- and HTT-dysfunction, including Huntington’s disease and many cancers. Building on our foundational discovery that, in addition to chromatin, SETD2 methylates microtubules, we asked if SETD2 had other novel targets. This led to the discovery SETD2 methylates the actin cytoskeleton as part of an HTT complex that controls actin dynamics and cell migration. Knockdown of HTT or the adaptor protein HIP1R inhibited actin methylation, with a corresponding defect in actin polymerization and migration, without affecting SETD2 methylation of histones or tubulin. In the case of SETD2 nuclear activity, the histone H3.3 lysine 36 to methionine (H3.3K36M) “oncohistone” mutation inhibits the ability of SETD2 to catalyze the H3K36me3 chromatin mark. We thus tested the effect of lysine-to-methionine mutations on SETD2 activity and localization. Expression of H3.3K36M inhibited histone methylation as previously reported, but had no effect on SETD2 cytoplasmic localization or cytoskeletal methylation. A corresponding tubulin mutation (K40M) had no impact on the ability of SETD2 to methylate microtubules or chromatin, effectively rendering the cell wild-type for SETD2 function. In contrast, expression of a pathogenic HTT construct containing multiple polyglutamine repeats - known to form protein aggregates in both the cytoplasm and nucleus - inhibited methylation of actin, tubulin, and histones. These findings, using substrate or binding partner mutations as tools to resolve the activity of a chromatocytoskeletal remodeler, reveal both the cellular compartment and target substrate act as determinants of SETD2 activity.

P1291

Autocrine insulin pathway signaling regulates actin dynamics in cell wound repair
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Cells undergo daily injuries, including those from mechanical and chemical stresses, that often damage the plasma membrane and underlying cytoskeleton. The molecular mechanisms deployed to repair such damage and avoid cell death are still largely unknown. Using the early Drosophila embryo as a genetically amendable model for single cell wound repair, we performed microarray analyses and drug inhibition studies to assess the contribution of gene expression. We find that the initiation of cell wound repair is dependent on translation, while transcription is necessary for later steps in the repair process.
From the microarray analysis, we identified 80 up-regulated genes and 173 down-regulated genes, totaling 253 genes whose expression was changed in response to laser wounding. We validated the top 15 up- and down-regulated genes respectively using RNAi knockdown and found that the wounded knockdown embryos exhibited disruptions at various post-initiation steps of the cell wound repair process, including wound over-expansion, delayed/altered rates of wound contraction, aberrant actin dynamics (premature actin ring disassembly, failure of actin ring disassembly, and/or accumulation of actin inside the wound), and/or remodeling defects. Interestingly, two of the top up-regulated genes are in the *Drosophila* Insulin-like signaling pathway. We find that this pathway controls actin dynamics through two downstream effectors: Girdin and Chickadee (profilin). We also find that disruptions throughout the pathway lead to abnormal wound repair. Our findings add to the understanding of how cells repair wounds, as well as provide new insights for wound repair in disease states.

P1292

Support cell actin dynamics and shape change during zebrafish hair cell death and regeneration

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Mammals and nonmammals often differ in their capacity to regenerate tissues after damage. For example, adult mammals are largely unable to produce new sensory hair cells, yet nonmammalian vertebrates can robustly regenerate hair cells throughout life. During hair cell regeneration, nearby gli-like support cells proliferate and differentiate into hair cells. During hair cell death, support cells extrude dying hair cells to maintain epithelial barrier integrity. It is unclear how dying hair cells signal to surrounding support cells to initiate repair and regeneration. We aim to characterize support cell shape change during epithelial repair and regeneration. We study zebrafish lateral line neuromasts, which are highly regenerative and easy to experimentally manipulate. To examine cell shape, we used a stable transgenic zebrafish line that labels the membranes of neuromast cells (*Tg:claudinb:lyn-GFP*). To study actin dynamics, we used CRISPR-Cas9 to create a transgenic line in which support cells express the F-actin sensor *LifeAct-GFP* (*Tg:atoh1a:LifeAct-GFP*). With these transgenic lines, we performed time lapse imaging to visualize support cell responses during hair cell death. Hair cell death was induced by applying neomycin tagged with Texas Red (neo-TR). We also ablated individual cells using transgenic zebrafish that express rat TrpV1 channels in hair cells, which causes specific cell death upon capsaicin addition. We found that neuromast support cells form multicellular F-actin rings that contract around the tops of dying hair cells, similar to structures reported in other animals. The F-actin rings formed around the time when neo-TR uptake spiked in hair cells, indicating that support cells respond quickly to hair cell damage. These support cell cytoskeletal dynamics are the earliest changes that occur after damage, and we hypothesize that they are linked to signals that promote hair cell regeneration.

P1293

Lasp-2 Regulates Protrusion and Adhesion of Chicken Primary Fibroblasts

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Lasp-2 is an actin-binding protein with a LIM domain, nebulin repeats, linker and an SH3 domain (Terasaki et al., 2004). Lasp-2 is localized in the actin network such as filopodia, lamellipodia and Z-lines. Lasp-2
also binds to paxillin, zyxin, and vinculin, the well-known components of focal adhesions via SH3 domain, but its biological implications have not been fully analyzed. We have previously shown that the N-terminal fragment of the LIM domain to the first nebulin repeat (LIM-n1) is the minimal actin-binding region of lasp-2, and the truncation of the LIM domain (lasp-2DLIM) causes the loss of F-actin-binding activity (Nakagawa et al., 2009). Lasp-2DLIM failed to accumulate in any actin-containing structures in neuroblastoma but localized in focal adhesions in myoblastoma. These data suggested cell type might affect the localization of the fragments in the filopodia and lamellipodia. Here, we analyzed the localization of the full-length lasp-2 and various filopodia, lamellipodia and focal adhesions. Fragment of the LIM domain dispersed in the cytoplasm and the LIM domain to the first nebulin repeat localized in the lamellipodia and filopodia as neuroblastoma. Kymograph analysis of lasp-2 and paxillin, which is known to be localized in nascent adhesions (premature focal adhesions) showed they accumulated and dissociated in focal adhesions at the same time. It also showed that paxillin tends to form a clear peak of accumulation within focal adhesions earlier than lasp-2. Lasp-2DLIM infocal adhesions significantly reduced, but small amounts of the fragment were observed. Lasp-2DSH3 only localized in matured focal adhesions. F-actin, paxillin, zyxin, and vinculin remained in the focal adhesions of fibroblasts expressing the lasp-2DLIM and lasp-2DSH3. However, the cell area decreased only in fibroblasts expressing lasp-2DLIM. Additionally, ratio of perimeter to area increased in lasp-2-expressing fibroblasts. The number and size of paxillin-positive focal adhesions decreased in fibroblasts expressing full-length lasp-2. In contrast, the number of focal adhesions containing zyxin, a component of matured adhesion increased. Lasp-2DLIM had no effect in the number and area of focal adhesions. These observations suggest the possibility that interaction between lasp-2 and components of focal adhesions promote maturation of nascent adhesion and increased protrusion fibroblast, and the effect need linking F-actin to focal adhesion.

P1294

Functional differences between β- and γ-actin: from translation dynamics to amino acid changes
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Two cytoplasmic acts in mammals - β- and γ-actin - differ only by 4 conservative amino acid changes at the N-terminus, however their nucleotide coding sequences differ by ~13% due to silent substitutions. These two acts show major functional differences: β-actin, unlike γ-actin, is essential for organism’s viability. The exact source of these differences are not well understood. We previously showed that introducing 5 point mutations into mouse Actb gene (β-actin) to encode γ-actin protein while keeping the β-actin nucleotide sequence nearly intact (Actbc-g) does not affect mouse viability, despite the lack of β-actin protein. This result demonstrates that β-actin’s requirement for viability depends on its nucleotide, rather than amino acid sequence. However, the specific amino acid sequences of β- and γ-actin are highly conserved among all vertebrates, indicating high evolutionary pressure on these changes in vivo. Here we investigated the specific determinants of β- and γ-actin function at the nucleotide and amino acid level by examining cell and mouse models expressing these acts and their codon switched variants. Using live cell translation measurements, we show that β-actin translation
elongation is faster than γ-actin. This difference, arising from the silent substitutions in their nucleotide sequences, leads to profound effects on cell migration rates and cell-substrate adhesion. At the same time, detailed phenotypic analysis of Actb-c-g mice, which lacks β-actin protein while retaining the nearly intact β-actin gene, reveals consistent defects in actin-rich cellular structures, especially the microvilli in the small intestine and the retina, where these structural defects result in reduced light sensitivity. Biochemical analyses suggest these defects arise from altered affinity of specific actin binding proteins (ABPs) for actin in Actb-c-g tissues, suggesting isoform specificity in these proteins' interaction with actin. Our data suggest that actin isoforms' N-termini co-evolved with ABPs to perform their functions in the cell. Together, our results shed light on the specific roles of nucleotide and amino acid sequence in actin isoform regulation: from silent substitutions to highly conserved amino acid differences.

**Autophagy**

P1295

**Autophagy insufficiency perturbed definitive hematopoiesis in zebrafish model**

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Autophagy is a highly conserved lysosomal degradation and recycling system of damaged organelles under various physiological conditions and stimuli, initiated through the activation of uncoordinated-51 like autophagy activating kinase (ULK1). Autophagy plays key roles in hematopoietic stem cell (HSC) maintenance and protects HSCs from cellular stress, but its role in definitive hematopoiesis is yet to be elucidated. Taken advantages of the robust and transparent zebrafish (*Danio rerio*) model, genome editing and pharmacological approaches, here we impeded autophagy by conditionally knocked out ulk1b (human ULK1 ortholog) to observe hematopoiesis. Lack of autophagy in zebrafish embryos by ulk1b knock out resulted in indecent conjugation of delipidated cytosolic microtubule-associated protein 1A/1B-light chain 3 (Lc3I) with phosphatidylethanolamine (PE) to generate lipidated LC3II. Autophagy inhibition further suppressed autophagosome (Lc3 puncta per cell) and autolysosome (co-localization of Lc3 puncta with the lysosome indicator dye, lysotracker red) numbers without affecting flux. Consequently, impaired autophagy dysregulated the hematopoietic lineages, which include ectopic expression of common myeloid progenitors (*pu.1*), pan-leukocytes (*l-plastin*), neutrophils (*mpx*) and macrophages (*mpeg1.1*) throughout definitive hematopoiesis. Contrarily, hematopoietic stem cells (*cmyb*), erythroid progenitors (*gata1*), embryonic hemoglobin (*hbae1.1*) and lymphoid progenitors (*rag1*) were significantly decreased in the caudal hematopoietic tissue (CHT). Furthermore, autophagy induction through calpeptin treatment was insufficient to ameliorate defective hematopoietic phenotypes in ulk1b mutants whereas autophagy inhibitor 3-MA analogously reacted with the autophagy deficient mutants. In general, our findings demonstrated that autophagy inhibition throughout zebrafish definitive hematopoiesis incorporates with atypical myeloproliferation, lymphopoiesis and erythropoiesis. Taken together, these findings reveal insightful roles of autophagy in hematopoietic cell fate determination.
CRISPR gene-engineered CYBB$^{\text{ko}}$THP-1 cells highlight the crucial role of NADPH-induced reactive oxygen species for regulating inflammasome activation through autophagy and autophagy-related processes

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CRISPR gene-engineered CYBB$^{\text{ko}}$THP-1 cells highlight the crucial role of NADPH-induced reactive oxygen species for regulating inflammasome activation through autophagy and autophagy-related processes

Daniela Stanga Ph.D$^{1,2}$, Fabien Touzot MD, Ph.D$^{1,2,3}$ The CYBB gene encodes the cytochrome b-245 which is a subunit of the NADPH oxidase enzymatic complex 2 (Nox2). This complex plays an essential role in the immune system by converting oxygen to reactive oxygen species (ROS). ROS kill foreign invaders and prevent them from reproducing in the body, thus fighting infection and preventing illness. Loss of function mutations in CYBB are associated with Chronic Granulomatous Disease (CGD), a rare primary immune disorder characterized by an increased risk of infections toward various bacterial and fungal pathogens. CGD can cause life-long debilitating illness, increased mortality and high healthcare costs. Autophagy shapes the host immune response at multiple physiological levels. While its major function is the regulation of metabolism, autophagy plays an influential role in immunity in three important ways: (i) inhibiting the replication of pathogens, blocking the accumulation of damaged mitochondria, and (iii) preventing activation of the inflammasome, a molecular scaffold that forms in response to pathogens or danger signals. In addition to autophagy, there are a couple of important autophagy-related processes used by cells for mediating both innate and adaptive immunity: (i) LC3-associated phagocytosis (LAP) which is involved in the internalization and degradation of pathogens; and LC3-associated endocytosis (LANDO), a hybrid between endocytosis and LAP. Interestingly, activation of either one of these processes causes down-regulation of conventional autophagy, which suggests that LAP/LANDO and the conventional autophagy pathway are mutually exclusive. Both LAP and LANDO are dependent upon the successful recruitment of LC3 to the phagosome membrane in a redox-regulated manner. Thus, the presence of ROS produced by NADPH oxidase could be a pre-requisite for the proper function of these autophagy-related processes. Using a Nox2-deficient THP-1 cell line that we engineered through CRISPR-Cas9 gene editing we found that these cells display compromised autophagy and increased secretion of inflammatory cytokines. We therefore reason that these defects are the result of Nox2 deficiency, which inhibits both the LANDO and LAP pathways with the following consequences: (i) defect of pathogen clearance, causing defective recycling of inflammatory cytokine receptors; and a prolonged inhibition of autophagy, due to unresolved LAP/LANDO, that eventually leads to the accumulation of damaged mitochondria and further inflammasome activation.

Elucidation of human ATG9A structure and function, the only transmembrane protein of autophagy

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Autophagy is a highly regulated catabolic pathway for the lysosomal degradation of cytoplasmic organelles, protein aggregates and intracellular pathogens. The process of autophagy is carried out by a complex molecular machinery comprising the products of more than 30 autophagy-related genes (ATG). This machinery translates different stress signals into the formation of a double-membraned vesicle, the autophagosome, which engulfs cytoplasmic particles and subsequently fuses with lysosomes, where the autophagic cargos are degraded and recycled. ATG9 is the only transmembrane component of the core autophagy machinery and it is essential for survival. In all organisms, ATG9 localizes mainly to the trans-Golgi network (TGN), from where it is sorted into ATG9-containing vesicles for eventual delivery to forming autophagosomes. These vesicles are hypothesized to be the source of lipids for expansion and closure of the autophagosomal membrane. However, more than 15 years after the discovery of ATG9, the molecular function of this protein remains unknown. To close this gap in knowledge, we combined cryo-electron microscopy, biochemical and cell-based assays to characterize the structure and function of ATG9. First, we solved the structure of the human ATG9 isoform A (ATG9A) at 2.9 angstroms resolution, representing the first high-resolution structure for any ATG9 ortholog. Interestingly, the protein forms a symmetric homotrimer with a pore in the center and an intriguing internal network of branched cavities. The interface in ATG9A involves extensive domain swapping in the membrane, and the cytosolic domains form an extended helical platform, presumably for protein-protein interactions. Second, our structure-based computational simulations predict that ATG9A has membrane-bending properties, consisting with this protein mostly being found in small vesicles (30-50 nm) and at the tip of the forming autophagosome. Finally, we also generated an ATG9A-KO cell line by CRISPR/Cas9 that displays impaired autophagosome formation; these cells were used to test the functional importance of key residues identified in the structural studies and to investigate the role of ATG9A in lipids trafficking to the autophagosome. In conclusion, these findings provide the first molecular insights into the function of ATG9 in autophagosome formation, an essential step in autophagy progression.

P1298

**Regulation of autophagy-dependent skeletal muscle regeneration and strength by ARHGEF3**

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Skeletal muscle regeneration after injury is essential for maintaining muscle function throughout aging. ARHGEF3, a Rho-specific GEF, negatively regulates myoblast differentiation through Akt signaling independently of its GEF activity in vitro. Here, we investigated ARHGEF3’s role in skeletal muscle regeneration by creating ARHGEF3 KO mice. These mice exhibited indiscernible phenotype under normal conditions. Upon injury, however, ARHGEF3 deficiency enhanced the mass, fiber size and function of regenerating muscles in both young and aged mice. Surprisingly, these effects were not mediated by Akt, but by the GEF activity of ARHGEF3. Likewise, overexpression of ARHGEF3 inhibited muscle regeneration in a Rho-associated kinase inhibitor-sensitive manner. We further revealed that ARHGEF3 KO promotes muscle regeneration through activation of autophagy, a process that is also critical for maintaining muscle strength. Accordingly, in old mice, ARHGEF3 depletion prevented muscle weakness by restoring autophagy. Collectively, our findings identify an unexpected link between ARHGEF3 and autophagy-related muscle pathophysiology.
**Correlative light electron microscopy of autophagosome biogenesis**
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Macroautophagy is a crucial transport pathway of unwanted or harmful cellular material to the lysosome. The material is isolated from the cytoplasm by a membranous structure named the phagophore. When the phagophore closes it forms a double-membraned autophagosome. The autophagosomes then fuse with endosomes and finally with lysosomes where the isolated material is degraded and recycled into the cytoplasm. The origin of the phagophore membrane and the biogenesis of autophagosomes are long-lasting questions that are still not fully understood. Our goal is to image forming phagophores of nonselective and selective autophagy at different time points to establish the 3D structure of the phagophore assembly site and identify potential membrane donors that would supply lipids to the phagophore. To be able to locate early phagophores we established a Correlative Light and Electron Microscopy (CLEM) protocol that enables us to track the phagophore formation over time by live cell imaging of early autophagy proteins such as ATG13 and DFCP1. We then fix the cells and capture 3D electron micrographs. From 3D electron micrographs we segment out a 3D model of the phagophore structure and its surrounding organelles. Currently we have collected data on starvation induced autophagy, and on both selective autophagy of mitochondria (mitophagy) as well as SQSTM1/p62 aggregates (aggrephagy). From these datasets we have modelled phagophores at different stages of formation, visualizing the phagophore and surrounding organelles in 3D.

**The many shades of ER-phagy: from its cellular function to the regulatory signaling pathways**
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The endoplasmic reticulum (ER) is the largest organelle of the cell, adapting dynamically to cope with high demand of newly synthesized proteins. Protein misfolding eventually occurring in the ER might lead to protein aggregation and ER dysfunction. Mammals developed evolutionary-conserved quality-control mechanisms to prevent this scenario. ER-phagy is a novel identified autophagy pathway targeting ER fragments for lysosomal degradation. This process occurs through ER-phagy receptors, ER proteins that bind LC3/GABARAP proteins on the autophagic membranes, allowing the incorporation of ER fragments into nascent autophagosomes. To date the functions of ER-phagy and the signalling pathways regulating this process are still largely unknown. We have identified ER-phagy as a transcriptionally regulated process. We demonstrated that nutrient starvation induces ER-phagy via the activation of TFEB and TFE3 transcription factors, master regulators of lysosomal biogenesis and autophagy. Once in the nucleus, TFEB and TFE3 induce ER-phagy through the induction of the ER-phagy receptor FAM134B, that binds the autophagosome membrane-associated protein LC3 and delivers a portion of ER to the lysosome for degradation. In addition to provide a starvation response we demonstrated that ER-phagy has ER-quality control functions, that complement the ERAD pathway. ER-phagy selectively removes misfolded procollagen molecules from the lumen of the ER, preventing their luminal aggregation in collagen.
producing cells, such as chondrocytes, osteoblasts and fibroblasts. Mechanistically, we found that the ER chaperone CALNEXIN acts as co-receptor that recognizes misfolded procollagen (PC) molecules and interacts with the ER-phagy receptor FAM134B, which delivers ER fragments containing both CALNEXIN and PCs to lysosomes for degradation. Notably, cells from patients affected by collagenopathies show activation of the TFEB-FAM134B axis, suggesting a role for ER-phagy in the pathogenesis of collagen-related disorders. Unexpectedly, we also found that ER-phagy is also activated in chondrocytes during skeletal development. We found that the FGF signaling, a critical regulator of chondrocyte differentiation, by suppressing the insulin signalling pathway promoted the activation of the TFEB-FAM134B mediated ER-phagy. The genetic inhibition of FAM134B activity in chondrocytes impairs FGF-mediated protein secretion and dampens cartilage and bone formation in Medaka fish, suggesting a physiological role of ER-phagy during skeletogenesis. Taken together, these data unveil unexpected pleiotropic functions of ER-phagy and describe a signaling pathway allowing ER-phagy to respond to both metabolic and developmental cues.

P1301

SVIP is a Molecular Determinant of Lysosomal Dynamic Stability, Neurodegeneration and Lifespan
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Missense mutations in Valosin-Containing Protein (VCP) are linked to diverse degenerative diseases including IBMPFD, amyotrophic lateral sclerosis (ALS), muscular dystrophy and Parkinson's disease. Here, we characterize a VCP-binding co-factor (SVIP) that specifically recruits VCP to lysosomes. SVIP is essential for lysosomal dynamic stability and autophagosomal-lysosomal fusion. SVIP mutations cause muscle wasting and neuromuscular degeneration while muscle-specific SVIP over-expression increases lysosomal abundance and is sufficient to extend lifespan in a context, stress-dependent manner. We also establish multiple links between SVIP and VCP-dependent disease in our Drosophila model system. A biochemical screen identified a disease-causing VCP mutation that prevents SVIP binding. Conversely, over-expression of an SVIP mutation that prevents VCP binding is deleterious. Finally, we identify an SVIP mutation in a human patient and confirm the pathogenicity of this mutation in our Drosophila model. We propose a model for VCP disease based on the differential, co-factor-dependent recruitment of VCP to intracellular organelles.

P1302

Replication stress induced nucleophagy targets nucleolar proteins and stabilizes the rDNA array
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Cell division is a highly coordinated process that ensures each cell receives an identical copy of the genome. Replication stress that arises from transiently stalled replication forks, due either to intrinsic cellular states or extrinsic clinical interventions (e.g., chemotherapeutics that block nucleotide production) increase the likelihood of tangled sister chromatids that form chromosome bridges in anaphase. Failure to properly resolve tangled sisters can lead to chromosome damage as well as packaging of chromosomes into abnormally formed nuclear structures called micronuclei. A high
incidence of micronuclei can lead to further chromosome damage in subsequent cell divisions and activate innate immune inflammatory responses. Though micronuclei occur at high frequency in cancer cells, they are less common in normal cell division and it has been suggested that surveillance pathways, including but not limited to macroautophagy, might be involved in eliminating micronuclei. To test this hypothesis, we monitored autophagic flux in budding yeast cells during replication stress and also after cells proceed into anaphase. We find depletion of nucleotides or PolI (DNA polymerase) activate autophagic flux during S phase arrest; flux persists after DNA replication has been allowed to complete and peaks as cells enter anaphase. This form of autophagy requires the intra-S-phase checkpoint kinases and our evidence suggests it involves both macroautophagy and microautophagy mediated targeting of the nuclear structures. The nuclear-ER (endoplasmic reticulum) macroautophagy receptors, Atg39 and Atg40, become enriched at nuclear-vacuolar junctions in a Vac8-dependent manner and form part of a large multi-protein assembly required to mediate both macro- and microautophagy pathways that target nucleolar proteins to the vacuole (e.g., Fob1, Nop1 and Net1, but not histones). We refer to this pathway as replication stress induced nucleophagy, or ReSIN. In cells defective in ReSIN, the rDNA arrays exhibit instability under chronic replication stress. We propose a model where ReSIN is part of a homeostatic pathway that preserves nucleolar state in the presence of partially replicated rDNA.

P1303

Phosphorylation of Nix Attenuates Myocyte Mitophagy
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Lipotoxicity is a form of cellular stress caused by the accumulation of lipids resulting in mitochondrial dysfunction and insulin resistance in muscle. Previously, we demonstrated that Nix, a lipotoxicity-responsive gene, accumulates in response to diacylglycerols induced by high-fat and sucrose (HFS) feeding and exacerbated by exposure to gestational diabetes (GDM) during fetal development. Here we identify a novel phosphorylation residue, activated by cilomilast treatment that can prevent Nix-induced mitochondrial dysfunction in muscle cells. In a series of gain- and loss-of-function experiments in rodent and human myotubes, we demonstrate that Nix accumulation triggers mitochondrial depolarization, fragmentation, calcium-dependent activation of DRP-1, and mitophagy. In addition, Nix-induced mitophagy leads to myotube insulin resistance through activation of mTOR-S6K inhibition of IRS-1. Through detailed phospho-peptide mapping of Nix, we identified a novel phosphorylation residue within the transmembrane domain, modulated by PKA activating agents, such as adrenergic agonist clenbuterol and the phosphodiesterase-4 inhibitor cilomilast. Treatment of myotubes with these agents serves to prevent Nix-induced mitochondrial dysfunction and restore insulin sensitivity. Furthermore, Nix knock-down or clenbuterol/cilomilast treatment rescued palmitate-induced phosphorylation of Ser1101 on the insulin receptor substrate-1 (IRS-1) and prevented insulin resistance. These findings provide insight into the role of Nix-induced mitophagy and muscle insulin resistance during an overfed state. Finally, our data supports the hypothesis that Nix regulates mitochondrial metabolism and insulin
signaling in myotubes and suggests a mechanism by which pharmacological activation of PKA may circumvent the mitochondrial dysfunction characteristic of insulin resistance.

**Bioengineering of Cell-Matrix Interactions**

**P1304**

**Nanopatterned Scaffolds Based on Bioresorbable Polymers and Graphene Oxide Induce the Aligned Migration and Accelerates the Neuronal Differentiation of Neural Stem Cells.**

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**AIMS:** Nerve lesions and injuries in the central nervous system have a strong impact on high financial expenses and quality of life for the individual patients. This research aims to develop and characterize a nanostructured polymer scaffold based on bioresorbable elastomeric co-polysteres functionalized with graphene derivatives to promote the attachment, integration and alignment of neural stem and progenitor cells without the need of extracellular matrix. **METHODS:** Scaffolds of lactide and caprolactone based copolyesteres were first nanopatterned with gratings of 300 nm linewidth and subsequently functionalized with polydopamine, which acted as an adlayer for the final immobilization of GO. Murine NSCs were seeded on these scaffolds and after 3, 7 and 10 days, cells were fixed and immunostained for neuronal and glial markers. Furthermore, the interactions, cell-to-cell contacts and synaptic connections were analyzed by SEM. **RESULTS:** Murine NSCs instead of grow forming floating neurospheres, were able to sediment, align and elongate in the nanograting axis. Immunohistochemistry analyses to NSCs showed the persistence of both neuronal and glial markers when seeded on GO-functionalized nanostructured scaffolds compared with the control. **CONCLUSIONS:** The combination of a nanostructured bioresorbable polymeric scaffold together with the functionalization of the surface with GO enables a simple and scalable method for a spatial alignment and elongation of stem cells committed to neural fate with no need of extracellular matrix (e.g., laminin). **ACKNOWLEDGEMENTS:** UPV/EHU (COLAB19/03) and Government of the Basque Country (GIC 15/52, IT-927-16) and Spanish Ministry of economy and Competitiveness (RYC-2013-13450) for the financial support and Bikaintek for PhD grants.

**P1305**

**Fibronectin-incorporated liposomes accelerate extracellular matrix-based tissue regeneration**

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The unfolded states of fibronectin (FN) eventually induce the formation of the extracellular matrix, which is necessary to generate new tissues. Hence, for the utilization of FN in tissue scaffolding, the demand for a biocompatible material that is capable of stretching compact FN prior to cell delivery is increasing. In this study, we demonstrate that negatively charged small unilamellar vesicles (SUVs) is a qualified candidate for FN delivery due to the enhancement of FN functions in tissue regeneration. Our SUVs were proven to alter the conformation of surface-bound FN greatly. In vitro experiments revealed that the complex of FN-SUV remarkably elevated the attachment, differentiation and migration of fibroblasts. The potential utilization of this complex in wound healing is also described with a significant improvement in rats with ulcerative colitis (UC). Collectively, the results suggest that appropriately manufactured FN-SUV could be a novel material not only for wound healing but also for other therapeutic applications.

P1306

Stellate cells, identified using energy dispersive x-ray spectrometry, are located within the lacunae of a biodegrading hydroxyapatite implants.

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Cellular interactions with biomaterials and implant materials at ultrastructural resolutions can be challenging to investigate. Bone, cartilage, biomaterials, and implants can be difficult to embed in resin and section at thicknesses compatible with transmission electron microscopy (TEM), which also has a restricted field of view and sample area. While sample preparation is easier for scanning electron microscopy (SEM) and much larger areas of the sample can be studied, the signal differentiation between materials and biological tissue is often insufficient. Determination of the different components within the sample can be demanding, even when biological stains have been applied. We present the use of multi-colour electron microscopy using energy dispersive x-ray spectrometry (EDS) as a technique to enhance the interpretation of SEM data and provide additional analytical capabilities in the study of tissue-biomaterial interactions. We used SEM and EDS to examine the degradation of hydroxyapatite implants and subsequent growth of new bone using minipigs as an animal model. Tissue samples were fixed, embedded in resin and ground to a thickness of approximately 1mm before being stained using uranyl acetate and lead citrate. EDS data was collected with an Ultim Extreme detector on an SEM using accelerating voltages of between 5 and 10kV and a beam current of 1na or less. Data was collected either as single area scans or using large area mapping in AZtec. EDS was instrumental in the identification of cells within the hydroxyapatite implant, showing cells located within voids or lacunae within the implant material. It was not possible to identify the cells using electron signals alone. All the cells within the implant were stellate, a morphology usually observed in osteocytes embedded in bone matrix. EDS was also used to identify hydroxyapatite particles that were ingested by cells, determine regions of new bone growth, and revealed variations in nitrogen concentration (often used as a marker for peptides (1)) in areas immediately next to the implant. EDS provided substantial image data that facilitated interpretation of structure in addition to biologically relevant compositional information. (1) Pirozzi, N.M., Hoogenboom, J.P. & Giepmans, B.N.G. (2018) Histochem Cell Biol 150, 509-520
**Fabrication of myoblast-enclosing collagen gel fiber for tissue-engineered skeletal muscle**

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INTRODUCTION: Tissue-engineered skeletal muscle is expected to regenerate large-scale muscle damages. Tissue-engineered skeletal muscle needs to obtain high contractile force to recover mechanical function of skeletal muscle. Highly orientated myotubes in the tissue is required to achieve efficient contraction of myotubes. Moreover it is necessary to fabricate long fibril tissue-engineered skeletal muscle to mimic tissue structure of native skeletal muscle. We focused fabrication technique to construct fiber-shaped collagen gel mixed with myoblasts. It is expected that myotube orientation can be aligned by fabricating fiber-shaped construct. The purpose of this study was to develop the fabrication technique to fabricate the myoblast-enclosing collagen gel fiber. METHODS: The spinning solution was prepared by mixing type I collagen solution and C2C12 myoblasts. Cell density was 1.0×10^7 cells/ml. Collagen concentration was 2.4 w/v%. The spinning solution was extruded from the nozzle into DMEM high glucose supplemented with 10% FBS. The nozzle and DMEM were controlled at 37°C. Two kinds of nozzles were prepared, and inner diameter of thick nozzle and thin nozzle were 820 and 510 μm. After the myoblast-enclosing collagen gel fibers were cultured in DMEM with 10% FBS for 2 days with both ends fixed, the collagen gel fibers were cultured in DMEM high glucose with 7% horse serum for 12 days to enhance differentiation of C2C12 myoblast to C2C12 myotubes. Myoblast and myotube orientation were evaluated with a phase contrast microscope. Expression of myosin heavy chain (MHC) was observed with fluorescent immunostaining. Fiber contraction with electrical pulse was observed with a phase contrast microscope. RESULTS AND DISCUSSION: Initial diameter of the myoblast-enclosing collagen gel fiber for thick and thin nozzles were about 825 and 420 μm. Diameter of the myoblast-enclosing collagen gel fiber decreased with cultivation, and fiber diameter of those for thick and thin nozzles decreased to 511 and 185 μm respectively at 14 days of cultivation. C2C12 myotubes were aligned with longitudinal direction due to shrink of the myoblast-enclosing collagen gel fiber, and myotubes in the collagen gel fiber for thin nozzle were highly orientated. According to phase contrast observation and fluorescent immunostaining at 14 days of cultivation, differentiation of C2C12 myoblasts was confirmed by detecting MHC expression and aligned C2C12 myotubes was confirmed. The myoblast-enclosing collagen gel fibers for thin nozzle contracted synchronously with electrical pulse, whereas that for thick nozzle did not contracted. CONCLUSION: The myoblast-enclosing collagen gel fiber fabricated by the developed fabrication technique had highly oriented myotubes and contracted with electrical pulse.

**Curvature and Elastic Energy Minimization in Glia Mechanosensation Response to Scaffolds**

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Both the geometric and elastic properties of nanofibrillar scaffolds are known to affect cellular mechanosensation. In the present work, the energy minimization framework for the cellular response to curvature-induced stress proposed by Biton, et al. [1], which combines the synergistic aspects of
curvature and elasticity within a united framework, is experimentally investigated for a series of promising polylactic acid (PLLA) biodegradable nanofibrillar scaffolds. A new and novel automated nanofiber analysis algorithm that generates the nanofiber diameter and orientation distribution from atomic force microscopy (AFM) height images is coupled with force curve measurements and used to generate experimental values for radius of curvature $R$ and along-nanofiber elasticity $E$ as inputs for the energy model. Stress fiber orientation as a cellular response is investigated for cerebral cortical astrocytes (P2-8, rat) using immunocytochemistry to identify cytoskeletal proteins and atomic force microscopy to identify nanoscale morphological features. A correlation between energy minimizing configurations and observed cellular responses is presented. [1] Biton YY1, Safran SA. The cellular response to curvature-induced stress. Phys Biol. (2009) 6(4):046010.

Orienting Cell Division Axis and Measuring Forces in Mitosis using ECM Fiber Geometry

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During mitosis, cells undergo drastic shape changes from spread to rounded via loss of adhesions. In the rounded state, extracellular cues are transmitted through the actin-rich retraction fibers, which connect the mitotic cell to the underlying matrix, thus determining the orientation of the metaphase plate and daughter cell positioning. Studies on 2D substrates show that the retraction fiber distribution is regulated by the interphase isotropic adhesion localization. In contrast, cells in the body attached to ECM fibers have adhesions at their poles. Here, using suspended fibers generated by the non-electrospinning STEP (Spinneret Tunable Engineered Parameters) fiber manufacturing technique, we recapitulate in vivo focal adhesion organization in diverse cell shapes to interrogate their interplay in determining the orientation of the metaphase plate and cell division axis. Within aligned fiber networks, we achieve three elongated cell shapes: aerofoil attached to 1-fiber with aspect ratio (AR) ~ 9 and 2 focal adhesion clusters (FAC), and parallel shapes on 2-fibers (AR~ 9 and 4 FACs) and on multi-fibers (AR~ 5 with ≥ 6 FACs). Crosshatch geometries induced symmetric kite shapes (AR ~1), and 4 diagonal FACs. Using HeLa cells expressing Histone-H2B-GFP, we found that aerofoil shapes had the widest spread in division axis orientation, and their metaphase plates had significantly higher rotational movements. These observations, albeit contrary to intuition, can be linked to the low attachment on single fibers, owing to fewer retraction fibers. This might also explain why aerofoil shaped cells take significantly longer (68.4±3 minutes) times to complete mitosis (NEBD-anaphase completion) than other cell shapes (50-55 minutes). Interestingly, in the parallel (2-fiber) shape, rounded cells often became confined in-between the fibers causing the normally contractile inward fibers to deflect outward, thus providing an unique opportunity to measure forces. Using ‘Nanonet Force Microscopy’, we report the transient evolution of forces as cells transition from a contractile state in interphase to expansive rounding state during mitosis. Altogether, using ECM-mimicking fibers, we demonstrate the relative contributions of interphase cell shape and adhesion geometry in guiding cell division, while providing a complementary technique to measure mitotic cell rounding forces in situ, without the need of any external perturbation.
Cancer Stem Cell Gene Signatures

P1310

**TAZ Sustains Cancer Stem Cell Function in Breast Cancer by Transcriptional Repression of an Epithelial Differentiation Program Including Laminin 332 and the beta4 Integrin.**

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The goal of this study was to investigate the mechanism by which the Hippo pathway effector TAZ maintains the pleuripotency of breast cancer stem cells (CSCs), which exhibit a mesenchymal phenotype. To achieve this goal, we transformed immortalized mammary epithelial cells (HMT-S1) with a constitutively active TAZ vector (4SA-TAZ) and observed a mesenchymal transition and induction of CSC properties. RNA-seq was performed to gain insight into the mechanism involved. We noted that active TAZ repressed expression of the beta4 integrin, its ligand laminin (LM) 332 and genes associated with a basal epithelial phenotype. These data suggest that repression of an epithelial phenotype by TAZ is necessary for the genesis of CSCs. This hypothesis is supported by the finding that re-expression of the beta4 integrin in TAZ-transformed HMT-S1 cells resulted in an epithelial transition. At a mechanistic level, we observed that TAZ and Zeb1 function to repress transcription of the LAMC2 subunit, a component of LM332. This key finding was substantiated by demonstrating that down-regulation of either TAZ or Zeb1 in a mesenchymal breast cancer cell line, BT549, or freshly established organoids from human breast tumors was sufficient to induce LAMC2 expression. We propose from these studies that one component of the mechanism by which TAZ sustains a mesenchymal phenotype and CSC function is to engage in a TAZ/TEAD/Zeb1 complex that represses LAMC2 and possibly the beta4 integrin. Moreover, the possibility exists that this complex represses other epithelial genes.

P1311

**Identification of a quiescent stem cell gene network in Glioblastoma using data mining and data analytics**

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Glioblastoma (GBM) is an aggressive type of brain tumor with low survival rate. Accurate diagnosis and tumor classification by type is critical for successful treatment. Stem cells exist as actively proliferative stem cells and resting non-proliferative quiescent stem cells. ‘Cancer stem cell theory’ purports that both stem cells drive tumor growth and determine tumor type, while quiescent stem cells cause recurrence of tumor after treatment. Proliferative and quiescent stem cells are well studied in non-tumor cell culture systems but due to their small number in tumors, it has been challenging to study them in cancer. To identify representative proliferative and quiescent stem cell genes, differential gene expression analysis algorithms were applied on non-tumor stem cell gene expression data mined from NIH/GEO. Five pairs of proliferative and quiescent stem cell genes were detected from five mined datasets. To identify gene expression patterns or networks associated with GBM, network construction algorithms were applied on cancer gene expression data mined from NIH/GEO. A total of eight GBM associated gene networks or modules were identified. Overlaying proliferative and quiescent stem cell gene lists on GBM modules revealed a quiescent stem cell gene enriched GBM module.
(HuAgeGBsplit_18). Logistic regression model built with quiescent stem cell genes (GSE70696_QNPbyTAP) present in HuAgeGBsplit_18 GBM module, was sufficient to distinguish between control and GBM samples. Taken together these results suggest that quiescent stem cell genes in GBM module identified here can potentially be used for diagnostic gene expression panels. To drive discovery and innovation all computational pipelines from this work have been made open source and customizable for application in other studies.

https://github.com/smukher2/GithubScientificReportsGlioblastomaStemApril2020

Footnote: This computational research was conducted by author using free open access resources (NIH/NLM PubMed Central, NCBI SRA/NCBI GEO and NSF Cyverse Discovery Environment), personal resources and personal funds. This work is self-owned (not affiliated with any organization/university).

P1312

A developmental cellular hierarchy in melanomauncouples growth and metastatic phenotypes

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Although melanoma is notorious for its high degree of heterogeneity and plasticity, the origin and magnitude of cell state diversity remains poorly understood. Equally, it is not known whether melanoma growth is supported by a subfraction of Melanoma Stem-like Cells (MSCs) and if so, whether MSCs and Metastasis-Initiating Cells (MICs) represent overlapping, interchangeable, or distinct cell populations. By combining single-cell gene expression profiling, multicolour lineage tracing and quantitative modelling, we developed a spatially and temporally resolved map of the diversity and trajectories of melanoma cell states during primary tumour growth and metastatic dissemination in a clinically-relevant spontaneous mouse model of melanoma. We show that melanoma growth and metastatic dissemination are fuelled, respectively, by two transcriptionally and spatially distinct melanoma subpopulations. Our findings implicate a hierarchical model of tumour growth that is supported by a population of cancer stem-like cells, which reside in a perivascular niche and exhibit a transcriptomic signature of pre-migratory neural crest cells established transiently during embryonic development. Metastatic dissemination is, instead, driven by a “mesenchymal-like” subpopulation, which preferentially accumulates at the invading front of primary lesions. We identified the transcription factor Prrx1 as a driver of the mesenchymal-like melanoma phenotype, and demonstrate that this population fuels metastatic dissemination to lymph nodes and distant organs through an EMT-MET-like continuum. These results will pave the way for the development of strategies that detect and, ultimately, intercept melanoma before its dissemination to vital organs.
P1313

Role of α-actinin 4 in breast cancer stemness maintenance
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Abstract: α-actinin-4 (ACTN4) is an actin-binding protein that regulates cellular motility, invasion and metastasis in invading cancer cells by modulating actin filament flexibility at the leading edge. Recently, we showed that in breast cancer ACTN4 regulates radioresistance, raising the possibility that it might be playing a role in cancer stemness. Here we address this question by first probing its levels in cancer stem cells (CSCs). First, we showed that in MDA-MB-231 breast cancer cells, ACTN4 is enriched in both CD44+CD24- CSCs which are mesenchymal in nature, as well as in CD44+CD24+ hybrid CSCs which retain a combination of epithelial and mesenchymal characteristics. Highest ACTN4 expression in hybrid CSCs was associated with highest motility as well as higher proliferation in these cells. Future experiments will probe the effect of actinin-4 knockdown on the CSC fractions. Key words: α-actinin-4, CSCs

P1314

Role of microRNAs that target stem cell (SC) genes in the emergence of tumor heterogeneity during colorectal cancer development
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Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in the United States and Worldwide. One reason that cancer therapeutics for advanced CRC is not curative appears to be due to tumor heterogeneity. Our research goal is to discover the molecular mechanisms behind tumor heterogeneity in CRC. We previously showed that CRCs contain multiple subpopulations of cancer stem cells (CSCs) which can explain tumor heterogeneity and resistance to treatment. To determine how CSC sub-populations might arise, we are studying microRNA (miRNA) expression in CRC SCs. MicroRNAs contribute to the process of tumorigenesis by regulating gene expression, and serving as tumor suppressor genes and oncogenes. Hypothesis: Different miRNAs target specific SC genes, which leads to multiple CSC subpopulations and tumor heterogeneity in CRCs. Indeed, our miRNA expression profiling of normal and malignant ALDH+ human colonic SCs showed that miRNA92a targets the SC gene, LRIG1, and upregulation of miRNA92a leads to decreased LRIG1 expression. We also discovered that miRNA23b targets the SC gene, LGR5, and miRNA23b is upregulated in ALDH+ CSCs. We identified several other candidate miRNAs that are predicted to target CD166, ALDH1A1, and BMI1 SC genes, and are determining if these miRNAs contribute to the emergence of specific CSC sub-populations in CRCs. Thus, the finding that miRNAs control gene expression in ALDH+ CSCs, indicates that miRNA expression patterns may define human colon CSC subpopulations and contribute to tumor heterogeneity.
Role of HOX genes in the stem cell origin of colorectal cancer

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HOX genes encode master regulatory transcription factors that regulate stem cells (SCs), but their role in cancer etiology is under-studied. Aberrant expression of HOX genes occurs in colorectal cancer (CRC) and SC overpopulation drives CRC development. Our goal is to ascertain how HOX-regulatory mechanisms play a role in the SC origin of CRC. We previously reported that HOXA4 and HOXD10 along with aldehyde dehydrogenase (ALDH) and the other components of the retinoic acid (RA) signaling pathway are selectively expressed in human colon SCs. We postulate that the RA signaling that regulates HOX expression mainly occurs through ALDH+ SCs in the colon. **Hypothesis:** Specific HOX genes regulate growth and differentiation of SCs in human colon and HOX gene dysregulation leads to decreased differentiation and SC overpopulation. Indeed, preliminary data from our bioinformatics analysis shows that many HOX genes are overexpressed in CRCs, which parallels the overpopulation of ALDH+ SC that occurs during CRC tumorigenesis. We are now using immunohistochemical mapping to measure proportions of SCs and HOX-positive cells in normal and malignant human colon tissues. We are also evaluating the effects of all-trans-RA (ATRA) on HOX gene expression and on cellular proliferation and differentiation using CRC cell lines. Our preliminary data shows that ATRA treatment of HT29 CRC cells leads to reduced proliferation and decreased expression of select HOX genes. These results indicate that HOX genes, e.g. HOXA4 and HOXD10, are expressed in colonic SCs, and are regulated by RA signaling. Overall, the overexpression of HOX genes in cancer SCs appears to contribute to the SC origin of CRC.

Overdose Exposure of bFGF Converts iPSCs into CSCs

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Our current understanding of tumorigenesis is largely rooted in studies that focus on cancer stem cells (CSCs). The term "CSCs" refers to the stem-like features, the tumorigenicity and tumor maintenance abilities. Previously, our group has investigated a new insight of tumor initiation by developing a CSC model from iPSCs/embryonic stem cells (ESCs) cultured in condition media from cancer cell lines, as a rich source of tumor secreted factors such as interleukins, growth factors and so on. To analyze the components of the CM the gene expression profiles obtained from the GEO database were compared between different triple-negative breast cancer cell lines. As the result, high expression of basic fibroblast growth factor (bFGF) was found one of the highest among the genes. Therefore, we assessed the influence of bFGF on the conversion of iPSCs. Chronic exposure to bFGF kept the miPSCs cells survived without leukemia inhibitory factor (LIF) while the cells failed to survive without LIF. Interestingly, CSC markers were found to be elevated after three weeks of bFGF treatment, and at the same time, cells acquired their self-renewal ability without bFGF and LIF. Our data suggest that chronic exposure of bFGF could have a pivotal role in the conversion of normal stem cells into CSCs. Collectively,
the tumor initiation could be explained by the wound healing effect which provides the production of overdose bFGF during the chronic inflammation.

P1317

**Evaluation of Breast Cancer Characteristics developed from Pluripotent Stem Cells**

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Breast cancer (BC) is the second common cause of death of women in worldwide. The malignancy and aggressiveness of BC has been correlated to the presence of breast cancer stem cells (CSCs). In this study, we used mouse induced pluripotent stem cells (miPSCs) with conditioned medium (CM) from different hormonal subtypes of BC cell lines, namely BT549 and T47D cells to generate a new model of CSC from miPSCs affected by the factors secreted from BC cells. These cells were transplanted in subcutaneous (S.C.) and in the mammary fat pad (MFP) of BALB/c nude mice. The formed tumors were excised for histopathological, immunohistochemical and immunofluorescence analyses as well as for primary cultures. Invasive lobular carcinoma was observed in MFP where miPS-T47DcmP1 cells were transplanted and ductal carcinoma in situ in MFP where miPS-BT549cmP1 cells were transplanted. While adenosis was observed where miPS-T47DcmP1 cells were subcutaneously transplanted, invasive ductal carcinoma where miPS-BT549cmP1 cells were subcutaneously transplanted. In the sections from MFP, estrogen receptor α (ERα), estrogen receptor β (ERβ), cytokeratin 8 (CK8), prolactin receptor, progesterone receptor (PR), green florescence protein (GFP), Ki67 and CD44 were immunohistochemical and immunofluorescence detected. The all the tumors and derived primary cells from exhibited the expression of ERα, ERβ, and CK8 by western-blotting and ERα, PR, Nanog, and CD44 by RTq-PCR. In conclusion, miPSCs were converted into CSCs under the environment of BC providing different malignant tumorigenic subtypes of BC.

P1318

**Single-cell epigenomic analysis reveals intestinal stem cells and uniquely enriched transcription factor binding motifs in colorectal adenomas**

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Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the United States. CRC often develops from a loss of function mutation on the adenomatous polyposis coli (APC) tumor suppressor gene, and this is a common developmental step for adenomas to become carcinomas. We first collected polyps and unaffected colon tissue from patients with documented germline APC mutations. Patients with germline APC mutations develop hundreds to thousands of polyps; this allows us to molecularly characterize multiple colon polyps within individual patients. Multiple polyps provide the opportunity to delineate chromatin accessibility profiles in dysplastic polyps of varying degrees. Through emerging single-cell technologies, we can elucidate genomic and chromatin changes at the single-cell resolution. We performed single-cell chromatin accessibility profiling (scATAC-seq) on 60 matched adenomatous
polyps and 20 adjacent normal tissues to illuminate genomic and epigenomic changes contributing to the development of CRC. Clustering of the scATAC-seq data reveals populations of immune, stromal, and epithelial cells. Within the epithelial cells, we identify two distinct populations of intestinal stem cells characterized by chromatin accessibility around LGR5+ and BMI1+ marker genes. Furthermore, performing a detailed bioinformatic investigation by calling and identifying marker peaks reveals that intestinal stem cell populations have enriched transcription factor binding motifs (TFBMs), including TCF/LEF, IRF, and Sp/KLF transcription factor families. Across patients and tissues collected, there are many TFBMs independently enriched for chromatin accessibility, suggesting the diversity of TFBM enrichment in adenomas. Recognizing these heterogeneous modifications may move us towards expertly detecting cancerous polyps at an earlier stage or distinguishing which pre-cancerous polyps have the potential to become malignant in colorectal cancer.

P1319

MAPK cascade inhibition along with increasing PI3K/AKT signaling activation convert iPSC Cells into cancer stem cells in the tumor microenvironment

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Cancer stem cells (CSCs) are suggested to be responsible for drug resistance and aggressive phenotypes of tumors. Mechanisms that regulating CSCs are still under investigation. Our lab has established a novel method to produce CSCs from iPSCs under cancer microenvironment using conditioned medium (CM) from cancer cell lines. By treatment iPSCs with CM, cells gain CSC characteristics. Recently, we showed that non-mutagenic chemical inhibitor compounds accelerate the conversion of mouse iPSCs cells into CSCs. Here, we treated iPSCs with different MEK/ERK pathway inhibitors for one week in the presence of a CM of Lewis lung carcinoma (LLC) cells and investigated the conversion process. We found that iPSCs acquired CSC phenotype by inhibition of the MEK/ERK pathway in the tumor microenvironment, CM from LLC, and the conversion was accompanied by enhancing the activation of the PI3K/AKT pathway. The AKT phosphorylation was found to be up-regulated in the converted cells which expressed stemness and cancer stem cell markers and maintained self-renewal ability. Therefore, The inhibition of MEK/ERK and enhancement of PI3K/AKT pathways resulting in the sustained stemness and acquiring the CSC phenotype and subsequent conversion of iPSCs into CSCs in the tumor microenvironment.

Cell Cycle Regulators of Development

P1320

On Mechanisms How Cell Size Regulates Zygotic Genome Activation

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During early development, cell size reduces exponentially and cell cycle elongates after a period of embryonic cleavage, accompanied with zygotic genome activation (ZGA) transcribing hundreds to thousands of zygotic genes essential for germ layers specification. Previous studies have established a
correlation between cell size, cell cycle and ZGA; however, the mechanisms of how embryonic cells coordinate the triad has been poorly known. Using metabolic labelling of nascent transcriptome and wholemount embryo confocal imaging on *Xenopus* early embryos that contain a gradient of cell sizes, we recently uncovered a new spatiotemporal patterning of ZGA dictated by cell size. To study cell size-dependent zygotic gene transcription, we developed a pipeline for sequencing all nascent zygotic RNAs from cells with 2-3 orders of magnitude variation in volumes at the animal pole (AP) and vegetal pole (VP) of blastula embryos, respectively. We discovered hundreds of zygotic genes whose activation following the spatial pattern of ZGA revealed by confocal imaging, i.e., first onset at the AP then followed by the VP, suggesting a cell size-dependent fashion of activation. Interestingly, these genes are enriched for specifying the presumptive ectoderm, suggesting a potential role of cell size in regulating ectoderm specification. Rapid cell cycles during cleavage stages prevent zygotic gene transcription and cell cycle elongation is dependent of cell size reaching a threshold. To understand how cell size and cell cycle regulates ZGA, we treated embryos at 5 hours-post fertilization (hpf), which is prior to large-scale ZGA, with cycloheximide (CHX), an inhibitor of protein translation, and assayed nascent transcriptome at 7.5 hpf when ZGA is initiated. CHX treatment halted embryo development, resulting in cell cycle arrest in interphase and increased cell volume as compared with control embryos. Intriguingly, the CHX-treated embryos exhibited active nascent transcription and most zygotic genes are actively transcribed at a similar level as in control embryos, suggesting that cell cycle elongation is sufficient to induce ZGA. This is consistent with a sizerm mechanism, but not a timer as CHX inhibits protein translation. To further elucidate the cell cycle effect on ZGA, we took advantage of a *slbp2* mutant of zebrafish embryos whose cell cycle progression is dramatically slowed due to reduced histones. Remarkably, zygotic gene transcription initiated significantly earlier in the *slbp2* embryos with slower cell cycle and larger cell size. Together, these data from different model organisms support a model in which cell cycle regulation underlies the cell size regulated genome activation and cell size may be important for regulating early cell fate specification.

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**A Spatial Gradient of Cell Size Regulates Genome Activation and Vertebrate Early Development**

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In early embryogenesis, embryos divide without growth. After the rapid reduction in cell volume, the embryos turn on nascent transcription in a process named zygotic genome activation (ZGA), in which development switches from maternal to zygotic control. ZGA is essential for subsequent gastrulation, but the pattern of ZGA and its implication on embryonic development is less studied. Through metabolically labeling of nascent transcripts, we have identified a spatial and temporal pattern of ZGA in *Xenopus* embryos that occurs initially in small cells at the animal pole and is delayed by two hours in cells of the vegetal pole. This spatiotemporal pattern tightly correlates with the gradient of cell sizes present in the blastula embryos, which is induced by the asymmetric cell division and the difference in division timings. To study the developmental importance of the evolutionally conserved cell size gradient and the resulting ZGA pattern, we developed an embryo temperature controller to eliminate or even reverse those patterns. It works by slowing down the division rate of the cells in the animal pole and speeding up the division of the vegetal cells. When setting different temperatures in animal poles...
and vegetal poles, cell division rates of each regions respond to the temperature accordingly. By generating *Xenopus* blastula embryos with reversed cell size gradients, we found that the spatial pattern of ZGA is also reversed; genome activation occurs first in the vegetal pole and is drastically delayed in the animal pole. Intriguingly, the ‘reversed’ embryos still have a similar threshold of cell size for genome activation onset, regardless of which the temperature gradient they have experienced. Those ‘reversed’ embryos tend to arrest in gastrulation as they fail to close the blastopore before neurulation. This work suggests that regulated spatial patterns of ZGA are important for coordinated embryonic development, particularly in blastula embryos that contain gradients of cell sizes.

**P1322**

**A non-epithelial function for planar cell polarity during lung morphogenesis**  
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The embryonic lung consists of a simple epithelial tube surrounded by mesenchyme that folds into a complex branched tissue. Following branching, the ends of these tubes expand into the saccule precursors of the alveoli, the specialized lung compartments that allow for gas exchange. Many well-studied pathways control lung epithelial development, but mesenchymal regulation remains poorly understood. *Vangl2*, a key component of the planar cell polarity (PCP) pathway that regulates convergent extension during gastrulation, has been implicated in both airway branching and sacculation. *Here, we mapped the role of Vangl2 and the epithelial PCP pathway in lung development.***

Immunofluorescence staining and single-cell RNA-sequencing analysis revealed that *Vangl2* is expressed by both the epithelial and mesenchymal compartments. In contrast, the expression of other PCP components, including *Celsr1*, is restricted to the airway epithelium. We found that *Vangl2* and *Celsr1* lungs branch in the expected pattern, but *Vangl2* lungs exhibit a slight developmental delay. Because *Vangl2* is expressed in both the epithelium and mesenchyme during branching, its absence in either or both of these tissues could cause the observed phenotype. To determine whether PCP is required in the epithelium, we used a tissue-specific Cre recombinase to delete *Vangl1/2* from the airway epithelium (epiCKO). We found that these lungs recapitulate the developmental delay of the *Vangl2* lungs. However, when cultured ex vivo, epiCKO, *Celsr1* lungs branch at similar rates. These data reveal that epithelial PCP is not required for airway branching morphogenesis. When we examined PCP-mutant lungs at later stages of development, we found that lungs from *Vangl2* embryos fail to form saccules. Surprisingly, both *Celsr1* and epiCKO lungs undergo normal sacculation, indicating that epithelial PCP is not required for sacculation. However, *Vangl2* is also expressed in mesenchymal cells during sacculation. By generating a lung mesenchymal knockout of *Vangl2*, we reveal that sacculation requires mesenchymal *Vangl*. Thus, our data show that *Vangl2* functions in a novel, *Celsr1*-independent manner to regulate distal lung morphogenesis. **References:** 1. Yates, L.L. et al. Hum Mol Genet. 2010. 19(11):2251-67.

**P1323**

**Cohesin Defects Disrupt Expression of an Essential Component of the CRL4 Ubiquitin Ligase Involved in the Thalidomide Teratogenicity Pathway**  
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Chemical or physical agents that disrupt proper development are termed teratogens. One such agent is thalidomide, a popular drug used in the 1950s for treating morning sickness. In utero exposure to thalidomide results in severe developmental abnormalities that include short limbs, mental disabilities, and organ malformation. Despite these effects, thalidomide is used today for the treatment of leprosy and cancer. Recent research identified a specific CRL4 E3 Ligase as the primary target of thalidomide, providing some insight into thalidomide’s molecular pathway. The extent to which genetic pathways contribute to developmental defects associated with CRL4 remains unknown. Intriguingly, Roberts Syndrome (RBS), caused by mutations in ESCO2, leads to developmental abnormalities that strongly resemble those of thalidomide babies. Importantly, Esco2 is an acetyltransferase that activates cohesins, which tether together distal DNA loci to form loops which are essential for proper gene expression. Mutations in cohesin give rise to Cornelia de Lange Syndrome (CdLS), a disease that leads to developmental abnormalities similar to RBS patients and thalidomide babies. We hypothesized that the phenotypic similarities between these three diseases could be due to a common target. Here, we used zebrafish embryos, a developmental model, to test the hypothesis that RBS, CdLS and thalidomide pathways are linked. Morpholino injections to individually knockdown (KD) Esco2 and Smc3, a cohesin subunit, produced overlapping phenotypes with those of zebrafish embryos treated with thalidomide. qRT-PCR was performed to assess transcript levels of cul4, ddb1, and crbn, genes that encode for components of the CRL4 complex targeted by thalidomide. Results showed significant downregulation of ddb1 in both Esco2 KD and Smc3 KD embryos. To unambiguously test if ddb1 expression is indeed downstream of Smc3, we performed rescue experiments in which Smc3 KD embryos were injected with ddb1 mRNA. Injection of ddb1 mRNA significantly rescued defects in body and eye sizes as well as abnormal otolith phenotypes that result from Smc3 KD. Interestingly, Esco2 KD phenotypes were exacerbated with ddb1 mRNA injections in Esco2 KD embryos. Injection of ddb1 mRNA significantly decreased body and eye sizes and an increase in abnormal otolith phenotypes was observed compared to Esco2 KD alone. These results are consistent with recent evidence that CRL4 targets Esco2 for degradation. In combination, given the core role of Ddb1 in CRL4 functions, and that CRL4 is a target of thalidomide, we posit that RBS and CdLS arise through CRL4 defects, similar mechanisms that give rise to thalidomide teratogenicity.

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An unexpected role for the LGN homolog AGS3 in promoting planar divisions during epidermal morphogenesis

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Asymmetric cell division, whereby progenitor cells divide to give rise to daughter cells that adopt different fates, is an important mechanism to promote cellular diversity and is essential for proper tissue morphogenesis. An evolutionarily conserved complex of polarity proteins directs asymmetric cell divisions, including the core scaffolding protein LGN (Gpsm2). The Drosophila LGN ortholog Pins plays a key role in both the positioning and orientation of the mitotic spindle in neuroblasts, ensuring the unequal inheritance of fate determinants that induces different daughter cell fates. In basal cell progenitors in developing stratified epithelia, we have shown that LGN localizes asymmetrically to the apical cortex and promotes perpendicular divisions. Epidermal LGN loss leads to elimination of
perpendicular divisions, decreased differentiation and impaired barrier function, resulting in neonatal lethality. This highlights the critical importance of maintaining a proper balance between planar (symmetric) and perpendicular (asymmetric) divisions. While the complex of proteins that promotes perpendicular divisions has been well characterized, much less is known about what orchestrates planar divisions. One candidate is the LGN homolog AGS3 (Gpsm1), although whether AGS3 possesses intrinsic spindle orienting activity remains controversial. Surprisingly, our data show that AGS3 does play an important role in spindle orientation in the developing epidermis, and in fact appears to oppose LGN function. Loss of AGS3 increases perpendicular (asymmetric) divisions, while AGS3 overexpression increases the proportion of planar divisions. However, because AGS3 does not appear to be detectable or polarized at the cell cortex in mitotic basal cells—as previously shown in neuronal progenitors—We hypothesize that AGS3 affects spindle orientation by regulating the apical recruitment of the mitotic spindle orienting complex. Supporting this, we observe that AGS3 knockdown or overexpression does not affect LGN cortical intensity, however LGN is mislocalized from the apical cortex. We also observed that LGN is epistatic to AGS3. Recently, we have described a phenomenon termed “telophase reorientation”, in which basal cells that enter anaphase at oblique angles correct to either planar or perpendicular during telophase. Using live imaging of epidermal explants, we show that LGN plays a maintenance role during telophase, promoting perpendicular reorientation. AGS3 also appears to function during telophase reorientation, as a higher percentage of oblique divisions reorient to perpendicular during telophase upon AGS3 loss. Together, these data suggest that the two vertebrate Pins orthologs play opposing roles in spindle orientation in the developing epidermis.

Chromosome Organization: Mammalian 2

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A Mechanism Underlying Weak Centromeric Cohesion in Cancer Cells

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Chromosome instability (CIN) is a major hallmark of cancer cells and believed to drive tumor progression. Several cellular defects including weak centromeric cohesion are proposed to promote CIN. However, the molecular mechanisms underlying these cellular defects are poorly understood. In a screening for SET protein levels in various cancer cell lines, we found that the majority of the cancer cells exhibit higher SET protein levels than non-transformed cells, including RPE-1. Cancer cells with high-level SET protein often show weak centromeric cohesion. Partial SET knockdown largely strengthens centromeric cohesion in cancer cells without obviously increasing PP2A activity, as revealed by distinct PP2A substrates. Pharmacologically increased PP2A activity in these cancer cells barely ameliorates centromeric cohesion. These results suggest that compromised PP2A activity, a common phenomenon in cancer cells, may not be responsible for weak centromeric cohesion. Furthermore, centromeric cohesion in cancer cells can be strengthened by ectopic Sgo1 overexpression and weakened by SET WT, not by Sgo1-binding-deficient mutants. Altogether, these findings demonstrate that SET overexpression is a major cause for impaired centromeric cohesion in cancer cells and illustrate mis-regulated SETSgo1 pathway as one of the underlying mechanisms.
The mitotic protein NuMA plays a spindle-independent role in nuclear formation and mechanics

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Most eukaryotic cells pack their genome into a single, round nucleus after mitosis, and failure to do so can compromise genomic integrity. How mammalian cells form such a nucleus remains poorly understood. NuMA is a long coiled-coil protein essential for building the spindle and its impaired function results in nuclear fragmentation. However, what role NuMA plays in nuclear integrity, or whether its perceived nuclear role stems from its spindle function, are unclear. Here, we show that NuMA plays a spindle-independent role in the formation of a single, round nucleus. NuMA keeps the decondensing chromosome mass compact at mitotic exit, and promotes a mechanically robust nucleus. NuMA's C-terminus binds DNA in vitro and chromosomes in interphase, while its coiled-coil acts as a regulatory and structural hub: it prevents NuMA from binding chromosomes at mitosis, regulates its nuclear mobility and is essential for nuclear formation. Thus, NuMA plays a long-range structural role throughout the cell cycle, contributing to the formation and mechanics of both the spindle and nucleus, two of the largest cellular structures.

NuMA Interaction with Chromatin is Vital for Proper Chromosome Decondensation at the Mitotic Exit

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Nuclear mitotic apparatus protein (NuMA) is an evolutionarily conserved large coiled-coil protein that localizes to the nucleus during interphase. However, in mitosis, upon nuclear envelope breakdown (NEBD), it gets released from the nucleus and localizes to the spindle poles and the cell cortex. Because NuMA is a highly abundant part of the mammalian nucleus (~10^6 copies), it was hypothesized to be a part of the structural framework of the nucleus. In this regard, the loss of NuMA's function based on antibody-based microinjections was associated with the impairment of nuclear architecture. However, since NuMA is essential for spindle assembly and maintenance, whether this phenotype was due to aberrant mitosis in the absence of functional NuMA remained elusive. In this study, by conducting fluorescence recovery after photobleaching (FRAP) and biochemical analysis, we uncover that NuMA is transiently bound to the chromatin in interphase in human cells. Moreover, we show that NuMA is localized on the condensed chromatin in prophase, and Cdk1/CyclinB1 activity is required to release it from the chromatin at NEBD. Notably, we identify evolutionarily conserved sequences rich in arginine and lysine at C-terminus of NuMA that are both necessary and sufficient for NuMA's interaction with the chromatin by directly associating it with the DNA. In the absence of such interaction, NuMA becomes significantly mobile in the nucleus, and lose its association with the chromatin in prophase. Cells expressing DNA-binding deficient mutant of NuMA are impaired considerably in chromatin decondensation at the mitotic exit. Additionally, the expression of the DNA-binding deficient mutant of NuMA causes the formation of abnormal shape nuclei. Furthermore, we discover that the effect of
mutant protein on nuclear architecture is not due to mitotic errors. But seemingly, it depends on its ability to polymerize into high-order structures such as puncta and fibrillar networks. Overall, this work links the chromatin binding ability of NuMA with the proper chromatin decompaction and maintenance of nuclear shape, and this function of NuMA appears to be independent of its role in spindle organization.

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**pre-rRNAs contribute to chromosome periphery protein recruitment and affect chromosome congression**

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**Background.** The chromosome periphery is a ribonucleoprotein layer that coats the outer surface of chromosomes during mitosis. This layer is composed by nucleolar proteins, precursor ribosomal RNA (pre-rRNA), DNA damage repair proteins, microtubule-interacting proteins and apoptosis regulators. The relocation of the nucleolar proteins to the chromosome periphery starts with Ki-67 exit from the nucleolus during late prophase followed by the other nucleolar components later in mitosis (between late prophase to anaphase). However, it is not well understood how this layer is organised or how these proteins are recruited to the chromosome periphery. We propose that pre-rRNAs participate in the recruitment of nucleolar components to the chromosome periphery after Ki-67 relocalises. **Results.** In order to decrease pre-rRNAs, we treated HeLa CDK1AS cells with a selective RNA polymerase I (RNAPI) inhibitor for a brief period in late G2 prior to release into mitosis. RT-PCR analysis of 45S and c-Myc (an RNAP II transcribed gene) transcript levels demonstrated that our treatment was affecting only RNAPI. Localisation of chromosome periphery proteins Ki-67, nucleolin, B23 and fibrillarin by immunofluorescence revealed that pre-rRNA downregulation disrupted the localisation of nucleolin, B23 and fibrillarin on the chromosome periphery during mitosis. Ki-67 levels were decreased, but its periphery localisation was clearly maintained. Previous reports showed that nucleolin depletion affects chromosome congression in HeLa cells. Thus, we performed an analysis of mitotic phases in the inhibitor-treated cells. We found that RNAPI inhibition resulted in an accumulation of prometaphase cells (almost 2-fold higher than untreated cells). **Conclusion.** These experiments suggested that pre-rRNAs help to organise the chromosome periphery downstream of Ki-67 recruitment, likely by promoting the recruitment of other nucleolar proteins such as nucleolin, B23 and fibrillarin. This has functional consequences in mitosis, as reflected by defects in chromosome congression and disjunction in anaphase.

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**Ki-67 clusters chromosomes during mitotic exit**

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During cell division, cells face the challenge to segregate their replicated chromosomes accurately into their daughter cells. Higher eukaryotes disassemble their nuclear envelope causing intermixing of nuclear and cytoplasmic components. Mitotic chromosomes are kept in suspension as individual bodies by the surfactant protein Ki-67, preventing them from coalescing with each other and allowing efficient interactions with the mitotic spindle. How during mitotic exit segregated chromosomes merge into a single nucleus remained unclear. We show that Ki-67’s properties radically change during mitotic exit. Ki-67’s molecular brushes collapse on the chromosome surface and in contrast to its surfactant function in early mitosis Ki-67 actively promotes chromosome clustering during anaphase, which is essential to clear the future nuclear area from large cytoplasmic particles, such as mature ribosomes. Thus, our study reveals how a single protein can dynamically change the surface properties of chromosomes, facilitating the re-establishment of nucleo-cytoplasmic compartmentalization after an open mitosis.

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Evolution of the chromosome proteome during synchronous mitotic entry
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Interphase chromatin is organised in part by cohesin complexes and also by interactions with the nuclear membrane and nuclear pores to allow regulated expression of the genome. This elaborate organisation is disassembled as cells enter mitosis and form individualised mitotic chromosomes to facilitate segregation of the duplicated genome. The transition from interphase chromatin to mitotic chromosomes is triggered by activation of several kinases, including CDK1 at the beginning of mitosis. Indeed, CDK1 activity is absolutely required to trigger the transition from G2 into prophase. Here, we investigated how chromatin is re-organised during the first steps of mitotic chromosome formation. We exploited a CDK1-dependent chemical-genetic system to trigger synchronous mitotic entry in chicken DT40 cells. Using the Chromatin Enrichment for Proteomics (ChEP) method, chromosomal proteins were crosslinked to DNA by formaldehyde and the chromatin fractions subsequently isolated under denaturing conditions from interphase and mitotic cells. The composition of proteins that were close enough to DNA to be crosslinked to it was then determined by mass spectrometry. We analysed the changes in abundance of proteins associated with DNA at 5 minute intervals over a period from G2 until after nuclear envelope break down (NEBD - i.e. early prometaphase). Hierarchical clustering revealed 38 different kinetic patterns among the 2547 proteins. Cluster size varied from 2 to 904. 1282 proteins (49.5 %) in four clusters went down (the proteins moved away from DNA) while 524 proteins (20.2%) in 11 clusters went up (the proteins moved onto the DNA). The remaining 756 proteins (29.2%) showed small (or no) deviations in either direction. The largest cluster, consisting of proteins that left the DNA at about the time of NEBD, was enriched for proteins involved in chromatin remodelling, histone modification, DNA repair and the DNA damage response. The first proteins to leave chromatin upon entry into mitosis were ribosomal RNA processing factors and mRNA splicing proteins together with some nuclear membrane proteins. Interestingly, RNA Pol I stayed longer on chromatin, accounting for the accumulation of unprocessed pre-rRNA that ends up coating the chromosome periphery later in mitosis. Two clusters containing 366 members account for all proteins known to associate with the chromosome periphery later in mitosis. These data constitute an important resource that provides a number of surprising insights into the behaviour of well-known complexes, including the outer kinetochore and the mitotic checkpoint complex.
Cilia and Flagella Assembly and Disassembly: Structure

**P1331**

**Live Cell Imaging Identifies Impaired IFT52 Trafficking and Dynamics in Motile Cilia from Mouse Tracheal Epithelial Cells with a Central Pair Defect**

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Defects in motile cilia, a critical organelle required for mucociliary clearance and fluid flow, result in the pediatric syndrome primary ciliary dyskinesia (PCD). The core, or axoneme, of the motile cilium is comprised of a 9+2 microtubule structure with nine outer microtubule doublets surrounding a central pair apparatus (CPA). While ciliary structure and function studies have historically been limited in mammalian cells due to technical challenges, we have used a novel application of high-resolution, live cell imaging to analyze protein trafficking and dynamics in the motile cilium on the surface of mouse tracheal epithelial cells (mTECs). Single particle tracking analysis of GFP-tagged intraflagellar transport protein IFT52 (IFT52-GFP) in mTECs from wild type mice and mice lacking CPA protein CFAP54, which have structural and functional ciliary defects, enabled quantitative comparison of protein velocity and behavior. In the absence of CFAP54, the rate of IFT52-GFP movement is impaired, and IFT52-GFP particles exhibit shorter average travel trajectories, demonstrating a constrained mobility in the cilia with a structural CPA defect. This study represents a novel and powerful application of live cell imaging to study protein dynamics in mammalian motile cilium and will enable future work aimed at understanding the cellular mechanisms underlying mammalian motile cilium and PCD pathogenesis.

**P1332**

**The Distal Appendage Protein CEP164 Is Essential for Efferent Duct Multiciliogenesis and Male Fertility**

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Cilia are evolutionarily conserved microtubule-based structures that protrude from the apical cell surface to perform diverse biological functions. Cilia are assembled on basal bodies (BBs), which are derived from centrioles and anchored to the plasma membrane via distal appendages (DAPs). Multiciliated cells (MCCs) are a specialized cell type with hundreds of motile cilia, lining the brain ventricles, airways, and reproductive tracts. Multicilia beat in a coordinated fashion to propel fluids/substances across the epithelial surface. Dysfunctional multicilia are associated with a wide range of diseases including hydrocephalus, airway infection, and infertility. In the male reproductive tract, MCCs are found in the efferent ducts (EDs), small tubules that connect the testis to the epididymis. Multicilia in the EDs move in a whip-like motion to stir the luminal contents and prevent sperm agglutination. CEP164 is a DAP protein that is essential for ciliogenesis. We demonstrated that CEP164
recruits a complex of Chibby1 (Cby1) and Cby1-interacting Bin/Amphipysin/Rvs (BAR) domain-containing (ciBAR) proteins to BBs to facilitate BB docking and cilium elongation. Mice lacking CEP164 in MCCs (FoxJ1-Cre;CEP164^{fl/fl}) show a significant loss of multicilia in the trachea, oviduct, and brain ventricles. Furthermore, we also noted male sterility, however, the precise role of CEP164 in the male reproductive tract remains poorly understood. Here, we found that the seminiferous tubules and the rete testes in FoxJ1-Cre;CEP164^{fl/fl} mice exhibit substantial dilation, indicative of dysfunctional multicilia in EDs. In addition, sperm accumulation and agglutination were frequently observed in seminiferous tubules and EDs. Consistent with these findings, multicilia were hardly detectable in the EDs of FoxJ1-Cre;CEP164^{fl/fl} mice although FoxJ1-positive cells were present. Immunofluorescence staining showed clear CEP164 signals at the base of multicilia in EDs. In FoxJ1-Cre;CEP164^{fl/fl} mice, the apical localization of Cby1, ciBAR, and BB markers was severely diminished, suggesting BB docking defects. TEM analysis of EDs is currently underway to confirm BB docking defects. Collectively, we conclude that removal of CEP164 in the MCCs of EDs causes BB docking defects and loss of multicilia, leading to sperm agglutination, dilation of seminiferous tubules and rete testis, and male infertility. Our study therefore unravels an essential role of the DAP protein CEP164 in male fertility.

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**TBCCD1: a key protein for basal body anchoring and microtubule network organization**

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Successful cilia assembly requires correct positioning and anchoring of the centrosome’s mother centriole/basal body (BB) to the cell membrane. Defects in primary cilia assembly/function cause severe diseases known as ciliopathies, typified by clinical manifestations, like infertility, obesity, brain problems, blindness, and kidney cysts. A clear picture of the different signals and players involved in centrosome positioning/anchoring is still not available. Published work from our group identified a new TBCC domain-containing human protein (TBCCD1). The depletion of TBCCD1 in human RPE-1 cells severely affects the relative position of the centrosome to the nucleus, and the efficiency of cells to assemble primary cilia. We aim to clarify the mechanisms involving TBCCD1 in centrosome/BB positioning and anchoring during ciliogenesis. Our recent data clearly shows that TBCCD1 is involved in centrosome microtubule (MT) anchoring and organization in RPE-1 cells and is required to normal localization patterns of acetylated MTs and centrosome structure. By depleting TBCCD1 in RPE-1 cells using siRNA, the structure of the sub-distal appendages is affected, causing the dislocation of the protein CEP170. Using a proximity-dependent biotin identification (BioID-MS) screen for interactors of both TBCCD1 splicing variants, we identified several BB anchoring and cytoskeleton organization proteins. Among the identified proteins by BioID-MS, there were several well-known proteins encoded by ciliopathy genes, e.g., centrosomal/centriolar satellite proteins OFD1, KIAA0753, and PCM1, which localization is affected
by TBCCD1 knockdown. Moreover, TBCCD1 is localized at the ciliary transition zone, a hotspot for the presence of ciliopathy-associated proteins. To clarify the role of TBCCD1 in cilia biogenesis, we used the ciliate *Paramecium tetraurelia*. In this organism, the complex cortex organization, basal bodies duplication, and positioning/anchoring are dramatically affected by TBCCD1 depletion. Taken all this into account, we propose that TBCCD1 is required for MT-anchoring and -organization activity at the centrosome, probably throughout interactions with some of its partners, with critical implications in basal body positioning/attachment to the cell membrane. This work was supported by Fundação para a Ciência e a Tecnologia (FCT), Portugal, to HS by project IPL/2019/MOONOFCLIL/ESTeSL and UIDB/00100/2020

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**Conventional actin is required for initial execution of the ciliary re-assembly program post-deciliation in*Chlamydomonas reinhardtii.*

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*Chlamydomonas reinhardtii*, a biflagellate unicellular green alga, is a powerful model for understanding human disease as its cilia closely resemble those in human cells. *Chlamydomonas* has two actin genes coding for a conventional actin (IDA5) and a novel actin-like protein (NAP1). Our previous studies show that after deciliation through acidic pH shock, cilia can be fully reassembled when either actin is present. However, when both actins are disrupted post-deciliation (by depolymerizing IDA5 with latrunculin B, LatB, in the *nap1* mutant background), ciliary reassembly is inhibited, and cilia reach half-length through incorporation of an existing pool of ciliary proteins, but not newly synthesized proteins. Surprisingly, our new results show that total actin disruption prior to and during deciliation causes an even more severe phenotype in which most cells completely fail to regrow cilia. Prior work in *Chlamydomonas* suggests deciliation is followed by a signal to the cell that it needs to upregulate the production of ciliary proteins to begin to rebuild cilia. Using semi-quantitative PCR, we found that normal upregulation of ciliary proteins still occurs, suggesting that the signal following deciliation is not disrupted by loss of actin. Our findings suggest that the presence of filamentous actin during deciliation is absolutely required for initiation of ciliary regrowth from already available protein even though they are not required for later stages of this incorporation and are dispensable for sparking transcriptional upregulation in response to deciliation

P1335

**Mechanism of primary ciliary resorption by phosphorylated Tctex-1**

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The primary cilia is a microtubule-based sensory organelle that is surrounded by an invaginated membrane structure, called the ciliary pocket (CiPo). The cilia is displayed at the cell surface during the G₀/G₁ phase in many cell types. Exposure of specific growth factors to the cilia promotes the ciliary resorption biphasically at the G₁-S transition and the G₂-M transition. Dysregulation of the ciliary dynamics is associated with hereditary disorders, called ciliopathy. However, regulatory mechanisms of the ciliary resorption have been poorly elucidated. Tctex-1, a light chain of cytoplasmic dynein complex,
has a dynein-independent role when it is phosphorylated at Thr94. We have shown that insulin-like growth factor-1 (IGF-1) is one of the most critical inducers of the ciliary resorption and IGF-1 accumulates phospho-(T94)Tctex-1 at the ciliary base, called the transition zone. In the present study, we aimed to investigate the mechanism of the ciliary resorption that was regulated by phospho-(T94)Tctex-1. An immortalized retinal pigment epithelial cell line (RPE-1) were serum-starved for 36 h to induce the ciliogenesis and re-treated with serum or 10 nM IGF-1 to promote the ciliary resorption. We found that phospho-(T94)Tctex-1 was responsible for Cdc42 GTPase activation, a key regulator of branched actin organization, in RPE-1 cells. We also found that phospho-(T94)Tctex-1 causes dynamin-, clathrin-, and Rab5-dependent CiPo membrane endocytosis through regulating the actin dynamics. Furthermore, to investigate the mechanisms of Cdc42 activation by phospho-(T94)Tctex-1, we identified a function unknown protein, Protein-X, in the cells. We found that knockdown of Protein-X suppressed the Cdc42 activity, endocytosis of the periciliary membrane, and the ciliary resorption in RPE-1 cells. In contrast, overexpression of wild type of Protein-X accelerated the ciliary resorption. These data demonstrate that the phospho-(T94)Tctex-1 drives the primary ciliary resorption by regulating Protein-X-dependent Cdc42 activation and CiPo membrane endocytosis. (References) Li and Saito et al., Nature Cell Biology, 13, 402-411 (2011); Yeh and Saito et al., Developmental Cell, 26, 358-368 (2013); Saito et al., EMBO reports, 18, 1460-1472 (2017); Saito et al., Bio-Protocol, 8, e2773 (2018)

P1336

Chromosome 21 represses primary cilia formation in a dose-dependent manner
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Down syndrome—a condition caused by an extra copy of chromosome 21—is the most common birth defect. An extra copy of chromosome 21 causes primary cilia formation and signaling defects in cultured cells, but the mechanisms for how chromosome 21 imbalances disrupt cilia are unknown. Pericentrin (PCNT) is a chromosome 21 resident gene that when elevated disrupts primary cilia formation and signaling. As a centrosome scaffold and trafficking protein, elevated PCNT increases interphase microtubules and disrupts protein trafficking to the centrosome and cilium. Using an RPE1 cell line engineered with three (Trisomy 21) or four (Tetrasomy 21) copies of chromosome 21, we explored the ciliogenesis and microtubule defects associated with chromosome 21 dosage. As found for cell lines isolated from individuals with Down syndrome, isogenic RPE1 cells with modified chromosome 21 copy number exhibit a dose dependent decrease in ciliogenesis. Moreover, elevated PCNT protein, resulting from Trisomy 21 and Tetrasomy 21, accumulates both at and around the centrosome as cytoplasmic foci. Elevated γ-tubulin, CEP215, and microtubule densities are also observed coincident with PCNT foci. These factors promote microtubule nucleation leading us to the model that elevated PCNT contributes to increased microtubule densities by recruiting γ-tubulin and CEP215 to the centrosome and cytoplasmic foci. We suggest that increased microtubule densities around the centrosome disrupt intracellular trafficking required to build the primary cilium. Consistent with this, reducing microtubule density by depolymerization with cold or nocodazole rescued ciliogenesis in Trisomy 21 and Tetrasomy 21 cells. These results suggest increased microtubule densities caused by elevated PCNT recruitment of γ-tubulin and CEP215 to the centrosome and cytoplasmic foci represses primary cilia formation and signaling.
Arp2/3 nucleated actin networks are required for ciliary assembly in *Chlamydomonas*

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The microtubule-dependent cilium has long been studied in the green alga *Chlamydomonas reinhardtii* due to its conservation of structures and assembly mechanisms relative to the cilia found on nearly all human cells. Our previous work found roles for actin in formation and trafficking of post-Golgi vesicles to cilia, as well as in the synthesis of ciliary proteins and organization of the gating region at the base of cilia. We have additionally tried to tease apart actin dependent ciliary functions and the nature of the actin filaments responsible for ciliary assembly by studying the Arp2/3 complex and branched actin. By using an inhibitor of the Arp2/3 complex, CK-666, and genetic mutants of the ARPC4 subunit of the seven-member Arp2/3 complex, we found that branched actin defective cells, similar to cells completely devoid of actin filaments, have defects in ciliary maintenance and dramatically impaired ciliary assembly. However, surprisingly, loss of Arp2/3 has a more dramatic phenotype for early incorporation of pre-existing ciliary proteins than for incorporation of newly synthesized proteins. Furthermore, we found that Arp2/3 interacts with both the conventional actin, IDA5, and the secondary, divergent actin found in *Chlamydomonas*, NAP1 to maintain cilium length and gating. Based on these data and others from our lab showing a role for Arp2/3 complex in membrane protein endocytosis, we hypothesize that branched actin plays a larger role in initial membrane remodeling for ciliary regrowth relative to post-Golgi trafficking of newly synthesized protein later in the assembly process.

Unprecedented views of visual cortex primary cilia in the neuropil from large TEM volumes provide cell-type specific insights

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Cilia in the brain project into a dense network of glial and neuronal processes, called the neuropil. Signaling through neuronal cilia influence both development and physiology. Unlike other tissues, there is not yet a pan-cilia marker that can be used to identify all of the cilia in the brain. Although TEM provides a label-independent strategy to identify and characterize primary cilia, extensive anatomical comparison of primary cilia across neuronal and glial cell-types has not previously been possible. In addition, the organization and demographics of processes in the neighborhood of primary cilia that influence signal release and perception are unknown. We repurposed three large volume connectomic TEM data sets to investigate primary cilia in the visual cortex. While cilia on excitatory neurons had stereotyped lengths and pia directed orientations, inhibitory neurons had more diverse cilia lengths and orientations. Despite these differences, neuronal cilia almost all docked at the plasma membrane, in contrast to astrocyte cilia that emerged from recessed pockets nestled deep in the plasma membrane. High resolution images also revealed that astrocyte cilia transition zones were much shorter than transition zones of neurons. Structural differences in cilia could be used to distinguish different cell types. Oligodendrocyte Progenitor Cells had additional distinctions, including short cilia and the
presence of intra-ciliary vesicles. Because large TEM volumes preserved cilia in the context of the tissue, we were also able to analyze the prevalence of astrocytic processes, dendrites, and axons adjacent to astrocyte and neuron cilia. Cilia concentrate receptors for molecules released from synapses, such as dopamine and serotonin, or from dense core granules, including neuropeptides like somatostatin. In the neuropil, many cilia passed adjacent to synaptic clefts. We also located dense core granules proximal to cilia. These new views in and around primary cilia in the brain inform our hypotheses about how cilia on different cell types in the brain perceive signals and influence physiology.

Directed Migration In Vivo and in 3D Environments In Vitro

P1339

A boost in mitochondrial energy production through a coordinated cellular program powers pioneer immune cell tissue invasion

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A coordinated cellular program to boost mitochondrial energy production powers pioneer immune cell tissue invasion. The ability of cells to move through challenging tissue barriers plays a fundamental role in the developmental establishment of tissue resident macrophages, the protective responses of immune cells, and the deleterious effects of cancer metastasis. Our lab seeks to employ the developmental migration of Drosophila macrophages as they penetrate the extended germband as a system to identify new mechanisms in migrating cells underlying tissue invasion. We find that a previously unexamined nuclear protein, CG9005, which we call Atossa, is induced and required in macrophages for their tissue invasion. Live imaging shows that the invasion defect in the atossa mutant is due to a delay in tissue entry and a decrease in the speed of only the first two macrophages, the invasion pioneers. RNA seq analysis shows that Atossa increases the mRNA levels of a helicase that we call Porthos and of several metabolic enzymes that can promote the formation of fuel for mitochondrial energy production. Each of these proteins is required for normal amounts of invasion and can partially rescue invasion when re-expressed at higher levels in the atossa mutant. Live imaging of macrophages with reduced levels of Porthos display a similar defect in pioneer cell invasion as that seen in the absence of Atossa. Polysome profiling reveals that the helicase Porthos enhances the occupancy on translating ribosomes of a set of RNAs with a conserved 5’ TOPL sequence, many of which are linked to mitochondrial metabolism and ATP production. Porthos depletion decreases the translation of a TOPL reporter, as well as mitochondrial oxidative phosphorylation as assessed by Seahorse. These findings along with our polysome profiling results argue that Porthos regulates the translation of mitochondrial OXPHOS complexes and thus energy production. This unusual control mechanism for a metabolic program appears to be evolutionarily conserved, as the two vertebrate orthologs of Atossa can each rescue the invasion phenotype, and are both enriched in immune cells. Our identification of a novel conserved nuclear factor that enhances mitochondrial energy production through concerted changes in the transcription and translation of metabolic enzymes and complexes of the mitochondrial OXPHOS pathway could shed light on how cells can increase their energy in other challenging contexts, even beyond immune cell responses.
Cytoskeletal dynamics during muscle progenitor migration

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Defects in cytoskeletal dynamics, cell cycle or morphogenetic movements are associated with many pathogenic processes. Here we explore the role of cytoskeleton regulation during cell migration and differentiation of mesodermal precursors in two distantly related taxa, the roundworm nematode, C. elegans and the zebrafish, D. rerio. The C. elegans sex myoblasts (SMs) migrate during larval development and differentiate into the adult vulval muscles. In zebrafish, the formation of new somites requires the continuous addition of new cells. Mounting evidence supports the hypothesis that these cells come from a population of bipotential neuromesodermal progenitors (NMPs) in the posterior tailbud of the embryo. NMPs undergo a two-step EMT and give rise to the migratory muscle progenitors, the paraxial mesoderm. The signaling pathways and guidance cues that lead the migration of these mesodermal precursors in both C. elegans and zebrafish have been established and both require FGF for proper migration. However, the genetic and kinetic mechanisms underlying their cell migration remain largely unknown. Using high-resolution sub-cellular microscopy, we analyzed the migration of these cells. We show that these cells generate both dynamic actin rich filopodia and blebs. Furthermore, using a cell cycle state sensor we establish the cell cycle state that these cells are in when they migrate. We show that the SM cells migrate in G0 while the paraxial mesoderm migrates in G2/S. We are currently working to establish the potential relationship between cell cycle and protrusive behavior using morphometrics analyses. We are poised to disrupt both key cytoskeletal regulators as well as the cell cycle to establish the relationship between the cytoskeleton, cell cycle and migration. These findings lay the groundwork for establishing C. elegans SMs and zebrafish paraxial mesoderm as a comparative model for cell migration and for studying the relationship between migration and cell-cycle state and cytoskeletal regulation.

Identification of a novel collective multicellularity independent of sorogenesis in the social amoeba Fonticula alba, a basal Opisthokont

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Social amoeba, which collectively aggregate, provide powerful models for studying origins of multicellularity. Asocial amoeba, Fonticula alba, was defined by genomic analysis not as Amoebozoa, but as a unique basal Opisthokont (Fungi and Metazoa branch). Despite its significant evolutionary placement, little is known about the biology of the organism. Fonticula alba undergoes vegetative growth cycle as single amoeba in co-culture of bacteria. Amoebae then enter a social cycle and form fruiting structures reminiscent of familiar Dictyostelid models, but mechanistically different. We identified new growth conditions that reveal a novel multicellular behavior. This organization, results in amoebae, which coalesce at the colony front to collectively invade new bacterial prey. The amoebae
organize into collective streams, which rapidly migrate outward and dynamically branch. This new social behavior is disconnected from the social cycle which drives fruit formation and has thus far not been described for Dictyostelids or other social amoeba. These two functional social cycles, one for arborized invasion and another for spore formation, have crucial implications in multicellularity, social feeding, cell-cell adhesion, collective invasion, hyphal growth, and Opisthokont diversification. These aspects make *Fonticula alba* a tantalizing model organism with unique biology for future evolutionary studies.

**P1342**

**Rules of contact inhibition of locomotion for cells on suspended nanofibers**

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Contact inhibition of locomotion (CIL), in which cells repolarize and move away from contact, is now established as a fundamental driving force in development, repair, and disease biology. Much of what we know of CIL stems from studies on 2D substrates that fail to provide an essential biophysical cue - the curvature of extracellular matrix fibers. We discover rules controlling outcomes of cell-cell collisions on suspended nanofibers, and show them to be profoundly different from the stereotyped CIL behavior known on 2D substrates. Two approaching cells attached to a single fiber do not repolarize upon contact but rather usually migrate past one another. Fiber geometry modulates this behavior: when cells are attached to two fibers, reducing their freedom to reorient, only one of a pair of colliding cells repolarizes on contact, leading to the cell pair migrating as a single unit. CIL outcomes also change when one cell has recently divided and moves with high speed- cells more frequently walk past each other. In collisions with division in the two-fiber geometry, we also capture rare events where a daughter cell pushes the non-dividing cell along the fibers. Our computational model of CIL in fiber geometries reproduces the core qualitative results of the experiments robustly to model parameters. Our model shows that the increased speed of post-division cells may be sufficient to explain their increased walk-past rate. Our results suggest that characterizing cell-cell interactions on flat substrates, channels, or micropatterns is not sufficient to predict interactions in a matrix - the geometry of the fiber can generate entirely new behaviors.

**P1343**

**Effect of simultaneous contact-guidance and chemotaxis cues on the 3D migration of breast cancer cells**

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Migration of cells is crucial in the physiological processes, such as embryogenesis and wound healing, as well as in the pathological conditions, such as cancer metastasis. The direction of migrating cells can be guided by chemical, physical, mechanical or electric gradients. In addition, cells can be guided by the ECM architecture features, as in contact guidance, where they move along the aligned ECM fibers. Most cells are simultaneously exposed not only to one, but to several guidance cues that can affect their migratory behavior. For example, cancer cells located in the perivascular niche are frequently exposed to the chemotactic cues, such as gradients of growth factors leaking from the blood vessel lumen, and
secreted by the adjacent stromal and immune cells. Simultaneously, they are also exposed to the aligned collagen fibers surrounding the blood vessel. To date, only a few multi-cue studies have been done, and specifically, studies that analyze effect of simultaneous chemotaxis and contact guidance are missing. Here, we expose the HS-578T breast cancer cells simultaneously to the gradients of growth factors and different levels of collagen fiber alignment, guiding the cells in either parallel or orthogonal direction compared to the chemotactic cue. Our preliminary data reveals that the parallel arrangement of chemical and contact cues significantly increases the speed and directionality of the cells when compared with single cues. The orthogonal arrangement shows no differences in the instantaneous speed of the cells when comparing to the values measured in cells exposed to single cues. In addition, we don’t observe the dominance of any of the two cues in the directionality of the cells. Notably, the persistence of the cells ranges in between single cue conditions suggesting that their migration in orthogonal signals is not dominated by one but both signals. Next, using a modified Hidden Markov Model (HMM) probabilistic algorithm to dissect every individual cell track into passive and active segments, we reveal the hierarchy that cells exposed to the orthogonally oriented cues follow while modifying their direction. In conclusion, our data shows that when the cancer cells are simultaneously exposed to both contact guidance and chemotaxis cues, both cues will impact the direction of cell migration. In addition, our data gives a possible explanation on how the multi-cue environment of the perivascular niche affects the dispersion of the motile cancer cells throughout the perivascular niche, maximizing the area of coverage and increasing the opportunities for future trans-endothelial migration.

P1344

Efficient 3D migration requires turning but not mechanical forces to navigate restrictive matrices
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Three-dimensional (3D) neutrophil migration through heterogeneous extracellular environments is crucial for immune surveillance and inflammation mediation. While much attention has been focused on inflammation cascade initiation, neutrophil recruitment also requires these cells to navigate the extravascular space’s narrow pores. In contrast to 2D migration on flat substrates, the chemotaxis in 3D physiological environments depends on the cells’ ability to remodel or deform the restrictive matrix. Unlike cancer cells, neutrophils do not seem to rely on metalloproteinase mediated degradation as a primary mechanism for matrix remodeling. Yet, neutrophils exhibit significant versatility in their migration, sustaining efficient chemotaxis index across various environments, including within hard-to-deform matrices. It is still unclear how neutrophils achieve this migratory function. We hypothesized that frequent, coordinated turnings of the cells could paradoxically give rise to efficient, long-range persistence when the cells cannot significantly deform their surrounding matrix. To test our hypothesis, we analyzed the trajectories of chemotactic neutrophil-like differentiated HL-60 cells at different length scales and quantified the 3D matrix deformations induced by the traction forces. We found that the cell’s ability to deform the matrix was reduced 10-fold when collagen concentration was increased from 0.25 to 2 mg/ml, and they adapted to this inability by turning more often to circumvent these narrow pores.
matrix pores. By examining the joint empirical distributions of the turning angle and chemotaxis angle, we observed that the neutrophils in stiffer, more impenetrable matrices turn more frequently than in softer matrices. However, their turns exhibit long-range coherence to keep the cells aligned with chemoattractant gradient. Specifically, we found cells used three consecutive turns on average to correct misalignments with the chemoattractant gradient irrespective of matrix density. We further found that this mechanism was independent of myosin-II contractility but required Arp2/3 activity. Using a minimalistic biased random walk model, we confirmed that turning characteristics alone can predict our experimental measurement of the cells' turning dynamics. We conclude that the ability to exert mechanical forces affect the rate of turning but not the chemotactic efficiency of neutrophils in 3D extracellular environment.

Host-Pathogen/Host-Commensal Interactions - 2

P1345

Distribution of StEXPAs and HRGPs-extensin changes as an effect of cell wall rebuilding in two types of PVY<sup>NNTN</sup>-Solanum tuberosum-interactions

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The plant cell wall acts not only as a physical barrier, but also as a complex and dynamic structure that actively changes under different biotic and abiotic stress conditions. The question is, how are the different cell wall compounds modified during different interactions with exogenous stimuli such as pathogens? Plants exposed to viral pathogens respond to unfavorable conditions on multiple levels. One challenge that plants face under viral stress is the number of processes required for differential cell wall remodeling. The key players in these conditions are the cell wall genes and proteins, which can be regulated in specific ways during the interactions and have direct influences on the rebuilding of the cell wall structure. The cell wall modifications occurring in plants during viral infection remain poorly described. Therefore, this study focuses on cell wall dynamics as an effect of incompatible interactions between the potato virus Y (PVY<sup>NNTN</sup>) and resistant potatoes (hypersensitive plant), as well as compatible (susceptible plant) interactions. Our analysis describes, for the first time, the expression of the potato expansin A3 (StEXPA3) and potato extensin 4 (StEXT4) genes in PVY<sup>NNTN</sup>-susceptible and -resistant potato plant interactions. The results indicated a statistically significant induction of the StEXPA3 gene during a susceptible response. By contrast, we demonstrated the predominantly gradual activation of the StEXT4 gene during the hypersensitive response to PVY<sup>NNTN</sup> inoculation. Moreover, the in situ distributions of expansins (StEXPAs), which are essential cell wall-associated proteins, and the hydroxyproline-rich glycoprotein (HRGP) extensin were investigated in two types of interactions. Furthermore, cell wall loosening was accompanied by an increase in StEXPA deposition in a PVY<sup>NNTN</sup>-susceptible potato, whereas the HRGP content dynamically increased during the hypersensitive response, when the cell wall was reinforced. Ultrastructural localization and quantification revealed that the HRGP extensin was preferably located in the apoplast, but deposition in the symplast was also observed in resistant plants. Interestingly, during the hypersensitive response, StEXPA proteins were mainly located in the symplast area, in contrast to the susceptible potato where StEXPA proteins were mainly observed in the cell wall. These findings revealed that changes in the intracellular distribution and abundance of StEXPAs and
HRGPs can be differentially regulated. The work was financed by National Science Center, Poland, NCN project numbers: 2019/03/X/NZ9/00499 and 2018/02/X/NZ9/00832

P1346

Virus-induced organelle remodeling viewed through the lens of Inter-ViSTA


All viruses rely on organelles to infect, replicate, and spread between host cells. Accordingly, viruses have numerous strategies for modulating organelle structures and functions, often leveraging virus-host protein interactions to remodel organelles into a pro-viral state. We set out to determine how mitochondria and peroxisomes - key organelles for metabolism and immune signaling - are manipulated during infection with cytomegalovirus (HCMV), a betaherpesvirus that stably infects nearly 90% of the world population. By combining quantitative mass spectrometry (MS) with confocal microscopy and molecular virology, we discover that HCMV rewrites peroxisome morphology, composition, and biogenesis during infection. This coincides with increased mitochondrial fragmentation and bioenergetics, altogether enhancing lipid metabolism for virus assembly. We next used immunoaffinity purification proteomics (IP-MS) to investigate virus-induced protein interactions at these organelles, focusing on the viral protein pUL37. As interpreting protein interactomes remains a major computational challenge for researchers, we developed the web-accessible analysis platform Inter-ViSTA (Interaction Visualization in Space and Time Analysis), which enables users to quickly build animated interaction networks that automatically integrate information on protein abundances, functions, complexes, organelle localizations, and temporal changes. With Inter-ViSTA analysis, we uncover temporally controlled interactions between pUL37 and integral mitochondrial and peroxisomal proteins. Further characterizing these associations via reciprocal IP-MS, microscopy, and genetic perturbations, we find that pUL37 engages key mitochondrial structural proteins at the timing of virus-induced fragmentation, including membrane fusion machinery and the highly conserved MICOS complex. We also examine interactions with apoptotic and calcium-sensing proteins, which reflect HCMV’s ability to simultaneously manipulate mitochondrial structure, antiviral signaling, and metabolism. Finally, we investigate associations with core components of the peroxisome fission pathway, showing that pUL37 controls peroxisome shape and numbers. Our work introduces Inter-ViSTA as a valuable tool for studying dynamic protein interactions and reveals strategies by which viruses restructure organelles to promote pro-viral functions.

P1347

Ehrlichia chaffeensisTRP120 Interacts With Notch EpidermalGrowth Factor Domains To Activate Notch Signaling

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Ehrlichia chaffeensis is a small, obligately intracellular gram-negative bacterium, and the etiological agent of human monocytotropic ehrlichiosis (HME), an emerging, life-threatening tick-borne zoonosis. E.
*chaffeensis* infects mononuclear phagocytes and has evolved molecular strategies to reprogram the host cell involving secreted effectors that interact directly with the host cell targets. Recently, we have shown *E. chaffeensis* evasion of innate defenses of the macrophage involve activation of Wnt and Notch signaling pathways. Interestingly, the *E. chaffeensis* tandem repeat effector, TRP120, has been shown to interact with host proteins important for activation and regulation of conserved signaling pathways including Wnt, Notch and Sonic Hedgehog. TRP120 directly interacts with ADAM17, a Notch metalloprotease involved in Notch activation, and FBW7, a ubiquitin ligase and Notch antagonist. In previous studies, we have demonstrated colocalization of TRP120 with both ADAM17 and the Notch-1 and activation of Notch signaling by TRP120. In eukaryotic cells, activation of Notch signaling occurs via ligand-receptor interactions through direct binding events at epidermal growth factor-like repeats (EGFs) in the extracellular domain (NECD) of the Notch receptor, specifically with the EGF 11-13 repeats. Hence, in this study we investigated the molecular interactions between *E. chaffeensis* TRP120 and NECD to determine the domains and motifs responsible for Notch activation. We identified homology between TRP120 and Notch ligands and determined that the TRP120-TR shares significant identity with known Notch ligands. To investigate direct interactions, we used surface plasmon resonance to show direct binding between TRP120-TR and NECD recombinant protein containing ligand interaction domain, EGFs 1-13. Using confocal microscopy, we further defined the TRP120-TR domain as the Notch activating domain. We are currently investigating peptide motifs within TRP120 with Notch ligand homology to identify a specific motif required for Notch activation. We have identified a TRP120-TR short linear motif (SLiM) required for Notch activation. Understanding the molecular basis of *Ehrlichia*-Notch ligand mimicry is important for understanding the survival strategies of intracellular pathogenesis. Defining such interactions may lead to the development of novel therapeutics that target host-pathogen protein-protein interactions.

**P1348**

**Characterizing the intracellular niche of human microsporidian parasite Encephalitozoon intestinalis**

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Microsporidia are a group of obligate intracellular pathogens related to fungi that cause lethal opportunistic infections in immunocompromised patients including AIDS patients. As such, microsporidia have evolved highly reduced genomes, having lost most protein coding genes for metabolic pathways and co-opt metabolites from the host cell for successful reproduction and development. To better understand the intracellular parasite niche, we are using both time lapse live-cell imaging and a high-throughput EM technique called serial block-face scanning electron microscopy (SBF-SEM). This allows us to characterize both the dynamics of parasite interactions with host cell organelles as well as define the physical interactions between the parasite and the intracellular environment of the host. For *Encephalitozoon intestinalis*, one of the most common host tissues is the mucosal epithelial lining of the intestinal tract. It is composed of multiple cell lineages that are organized into a unique 3-dimensional crypt-villus structure, which is not well modeled by 2D cultured cell lines. Therefore, to begin to understand the parasite niche during replication in 3D tissues, we have used mouse intestinal organoids to create an *in vitro* cell culture infection model representative of the cell type diversity and complex architecture of the *in vivo* tissue niche. This will give us insights into how a
human disease causing microsporidian parasite manipulates its hosts for successful growth and development.

P1349

_Cryptosporidium Parvum_ Infection Triggers Type I IFN Response in Intestinal Epithelial Cells through Host Delivery of CSpV1-dsRNAs

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_Cryptosporidium parvum_, a protozoan parasite that infects the gastrointestinal epithelium and other mucosal surfaces in humans and animals, is an important opportunistic pathogen in AIDS patients and one of the most common enteric pathogens affecting young children in developing regions. Recent advances have revealed a significant Type I interferon (IFN) response (e.g., induction of IFN-alpha and IFN-beta) in host epithelium following _C. parvum_ infection but the underlying molecular mechanisms are unclear. _Cryptosporidium parvum virus 1_ (CSpV1) is a member of the family _Partitiviridae, genus Cryspovirus_ that infects _C. parvum_. Using an _in vitro_ infection model employing IEC4.1 cells (transformed but non-tumorigenic murine intestinal epithelial cells) for genome-wide transcriptome (RNA-Seq) analysis, we demonstrated that several Type I IFNs were upregulated and a panel of Type I IFN-stimulated genes were significantly induced by _C. parvum_ infection. Induction of Type I IFNs and Type I IFN-stimulated genes was further confirmed in intestinal epithelium using a well-documented _in vivo_ model of neonatal mice. Interestingly, we observed the delivery of specific _C. parvum_ RNAs into infected host epithelial cells following invasion of the parasite. We found that the most abundant RNAs delivered into host cells from _C. parvum_ included two viral dsRNAs from CSpV1, CSpV1-dsRdRP and CSPV1-dsCA, as assessed by using PCR and _in situ_ hybridization. Host delivery of CSpV1-dsRNAs required direct parasite-host interactions and was not due to the nonspecific endocytosis (phagocytosis or macropinocytosis) by epithelial cells. Intriguingly, transfection of IEC4.1 cells with synthesized CSpV1-dsRNAs (either dsRdRp or dsCA) could induce significant expression of Type I IFNs and Type I IFN-stimulated genes. Transfection of siPOOLs to knockdown CSpV1-dsRNAs in IEC4.1 cells partially blocked the induction of Type I IFNs and Type I IFN-stimulated genes induced by _C. parvum_. Taken together, these data suggest that host delivery of CSpV1-dsRNAs may account for the Type I IFN response in intestinal epithelial cells following _C. parvum_ infection.

P1350

Determining the Role of Multiple Endocytic Pathways in Promoting _Listeria monocytogenes_ epithelial cell-to-cell spread

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_Listeria monocytogenes_ is a bacterial pathogen that is able to spread intracellularly, travelling between adjacent cells without exposure to the extracellular space. Recent studies have identified host cell factors that promote _L. monocytogenes_ cell-to-cell spread and have characterized the mechanisms by which some of these factors facilitate spread. Additionally, a population-level study has uncovered heterogenous spreading behavior, where some bacteria take small steps to spread to neighboring cells, while other pioneer bacteria travel farther. To follow up on an RNAi screen which identified cell junction
protein, E-cadherin, as a factor promoting *L. monocytogenes* cell-to-cell spread, we transduced A431D cells, which do not express endogenous E-cadherin, with wild-type E-cadherin or acyto E-cadherin that lacks E-cadherin’s cytoplasmic domain. Spread efficacy was greater between cells expressing WT E-cadherin than cells expressing acyto or no E-cadherin, suggesting that E-cadherin’s cytoplasmic domain contributes to spread. Moreover, *L. monocytogenes* was more likely to spread between two neighboring WT E-cad cells than from a WT E-cad donor to a null E-cad recipient cell. This leads us to conclude that E-cadherin’s cytoplasmic domain participates in spread at the recipient side of cell contacts. In particular, we proposed that E-cadherin promotes protrusion engulfment, where donor cell protrusions containing *L. monocytogenes* would be internalized into the recipient cell simultaneous with E-cadherin through a caveolin dependent process. In support of this hypothesis, we have evidence showing that blocking caveolin-mediated endocytosis with the drug filipin and preventing E-cadherin’s interaction with caveolin using the caveolin-scaffolding domain peptide, cavtratin, both reduce spread in WT E-cad cells but not acyto E-cad cells. Additionally, we uncovered a second endocytic pathway that contributes to spread, as inhibiting micropinocytosis with the drug EIPA reduced *L. monocytogenes* cell-to-cell spread in both WT E-cad cells and acyto E-cad cells. Furthermore, we found that inhibiting E-cadherin dependent and caveolin-mediated spread produced more compact bacterial foci, while inhibiting micropinocytosis did not alter focus shape. As reducing the number of pioneer bacteria that take longer steps compacts foci, we reasoned that *L. monocytogenes* cell-to-cell spread between immediate neighbors occurs via micropinocytosis, while pioneer bacteria employ caveolin to spread. Through this work, we have discovered additional mechanisms through which *L. monocytogenes* spreads from cell to cell and have connected molecular mechanisms describing spread between individual cells with the population-level growth of the bacterial focus over time.

P1351

**Defining Mechanisms Underlying Virus Regulation of Mitochondrial Bioenergetics During Infection**


Alterations of mitochondrial functions and cellular metabolism are hallmarks of nearly all viral infections. As obligate intracellular parasites, viruses rely on mitochondria for the production of biosynthetic precursors and energy necessary for generating new viral particles. The prevalent pathogen human cytomegalovirus (HCMV) alters both mitochondrial structure and metabolism during its replication. However, how HCMV induces oxidative phosphorylation remains unknown. Employing a multidisciplinary approach integrating virology, microscopy, and mass spectrometry-based proteomics, we discover that a previously uncharacterized viral protein, pUL13 targets the mitochondria and increases oxidative phosphorylation during infection. We establish that pUL13 is required for productive HCMV replication. We further demonstrate pUL13 is sufficient to increase cellular respiration, not requiring the presence of other viral proteins. By characterizing the temporal pUL13 functional interaction networks during infection, we discover and validate that pUL13 targets the MICOS complex, a critical regulator of mitochondrial architecture and electron transport chain (ETC) function. Our findings address the outstanding question of how HCMV modulates mitochondria to increase bioenergetic output and expands the knowledge of the intricate connection between mitochondrial architecture and ETC function.
Subcellular localization of effector proteins of the bacterial wilt pathogen in plant host cells
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Ralstonia solanacearum is the causative agent of bacterial wilt disease, which effects a wide variety of plants, including important crops such as potato and tomato. Ralstonia uses its type III secretion system (T3SS) to inject a suite of effector proteins into plant cells. The function of many of these effector proteins is unknown. Determining the subcellular localization of these effectors can provide insight into their functions. We determined the subcellular localization of a suite of Ralstonia effector proteins in a host plant Nicotiana benthamiana. We generated C-terminal GFP fusions to 14 effectors (RipB, RipD, RipH2, RipI, RipO1, RipS2, RipS3, RipV1, RipW, RipX, RipAF1, RipAM, RipAR, and RipV) from the Ralstonia strain GMI1000. These effector-GFP constructs were transiently expressed in leaves using Agrobacterium tumefaciens-mediated transformation, and epidermal cells of transformed leaves were examined with confocal microscopy. We found that the effectors localized to a number of cellular regions, including the nucleus and cell periphery, the nucleus alone, the peroxisome, and the plasma membrane. Plasma membrane localization was confirmed by plasmolysis experiments. Additionally, we identified one effector (RipAR) with punctate localization in cells. Time lapse imaging showed that puncta were mobile, generally moving along the cell perimeter. Our data indicate that effectors target a variety of regions within the cell, indicating that they may interfere with multiple host functions. We hypothesize that effectors that localize to the nucleus may interfere with transcription, while effectors that localize plasma membrane may interact with host proteins embedded there. Future work on effector function will allow for a better understanding of this pathogen and in turn inform treatments and control strategies for bacterial wilt disease.

Molecular mechanisms of antibiotic resistance in Pseudomonas sp. strains isolated from the intestine of rainbow trout, Oncorhynchus mykiss
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Most members of the Pseudomonas sp. are naturally resistant to a wide range of antibacterial drugs due to a complex of mechanisms. The most common mechanisms include low cell wall permeability to antibiotics, lack of specific targets, and accelerated efflux. Since a high percentage (67.4%) of Pseudomonas sp.polymorphs with biofilm-forming activity was found in O. mykiss intestines, it has been suggested that their ability to form biofilms could be potentially beneficial to resist to known antibacterial drugs. To probe this hypothesis, we studied the spectrum of antibiotic resistance of biofilm-forming Pseudomonas sp. strains isolated from the microbiota of aquaculture rainbow trout individuals manifesting bacterially derived disease symptoms. Totally twenty strains of Pseudomonas sp. with pronounced multi-resistance to the antibiotics, including aminoglycosides of the I and II generations, penicillins of the III generation, fluoroquinolones of the II generation, cephalosporins of I, II, and III generations, and chloramphenicol, were isolated and identified from the microflora of the trout.
The multi-resistance of isolated pseudomonad strains apparently resulted from the synthesis of exopolysaccharides forming biofilms. Since a high percentage of antibiotic-resistant strains (up to 72.7%) of pseudomonads, we supposed that their exopolysaccharide matrix could not only reduce the effect of the drug on microbial cells, but also led to a stable population of persisting host's cells. The research was supported by a grant from the Russian Science Foundation (project no. 20-66-47012) in partnership with Irkutsk State University.

**Immune system**

**P1354**

*Complete disruption of the macrophage checkpoint CD47:SIRPα increases phagocytosis of mAb-opsonized tumors, producing durable cures *in vivo* and *de novo* anti-tumor IgG*


The immune checkpoint between the receptor SIRPα on macrophages and CD47 expressed by all cells acts to inhibit phagocytosis of ‘self’ cells. Blocking this checkpoint in combination treatments of liquid tumors shows some efficacy clinically, but the durability of these responses and efficacy against solid tumors remain unknown. Anti-CD47 does not completely block CD47 in solid tumors, and so we generated a CD47 knockout B16 murine melanoma model, finding normal growth of B16 KO tumors in syngeneic C57 mice. Phagocytosis *in vitro* of KO cells opsonized with a mouse mAb against the melanoma antigen Tyrp1 was enhanced relative to mAb-opsonized B16 with wildtype-levels of CD47. Without opsonization, B16 CD47 KO or B16 CD47-Tyrp1 double-KO (DKO) were not engulfed. Intravenous administration of anti-Tyrp1 had no effect on wildtype tumors, whereas nearly half of mice with KO tumors survived, and most rejected later re-challenge with KO or DKO tumors. Survivors generated anti-B16 IgG of all subclasses against a broad set of antigens, based on immunoblotting and on equivalent binding to DKO and KO cells. The *de novo* IgG has the potential to overcome antigen escape in tumor evolution, which is problematic for many targeted therapies. Translatability is suggested by cures of the same model when treated by marrow monocytes with efficient blockade of SIRPα. These are the first studies of the widely used B16 solid tumor model to demonstrate durable cures based on CD47-SIRPα disruption.

**P1355**

*Cellular Mechanisms Of NETosis: From Microvesicle Shedding To Extracellular DNA Release*

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Neutrophil extracellular traps (NETs) are web-like DNA structures decorated with histones and cytotoxic proteins released by activated neutrophils. Initially described as a way for neutrophils to trap and neutralize pathogens during innate immunity, NETs are now widely implicated in the detrimental effect of several autoimmune diseases. Peptidylarginine deiminase 4 (PAD4) citrullinates histones and is required for NETs formation (NETosis) in mouse neutrophils. While various molecular mechanisms mediating NETosis are being revealed, the cellular events driving NETs release are still unclear. Here, we determined the sequence of cellular events in NETosis, and examined the role of PAD4 in these events. We performed high resolution time-lapse microscopy of mouse and human neutrophils (PMN) and differentiated HL60 neutrophil-like cells (dHL-60) labelled with fluorescent markers of the cytoskeleton and organelles and stimulated with ionomycin, lipopolysaccharides or C. albicans to induce NETosis. We found that stimulated neutrophils eject extracellular DNA after decondensation of the nuclear DNA in the nucleus, rupture of the lamina meshwork and nuclear membrane allowing the release of decondensed DNA to the cytosol, disassembly of the actin, microtubule and vimentin intermediate filaments networks, vesiculation of the endoplasmic reticulum and plasma membrane and finally rupture of the plasma membrane. More importantly, we found that these cellular pathways occur in a specific and well conserved temporal order suggesting a requirement for a precise sequence for progression through NETosis. Indeed, inhibition of actin disassembly, one of the first cellular events, blocked NET release. To examine the role of PAD4 in NETosis we isolated neutrophils from PAD4-deficient mice and generated a PAD4-knock down (KD) HL60 CRISPR line. We found that chromatin decondensation, lamin meshwork and NE rupture and extracellular DNA release required the enzymatic and nuclear localization activities of PAD4. Thus, NETosis proceeds by a step-wise and well conserved sequence of cellular events culminating in the PAD4-mediated expulsion of DNA. Our data further suggest that targeting these cellular pathways might be a better approach for controlling NETosis progression than targeting the various divergent signaling pathways. Indeed, microvesicle shedding, actin and lamin meshwork disassembly, DNA de-condensation, nuclear envelope and plasma membrane rupture are now potential therapeutic targets for NETosis inhibition.

P1356

**Restarting Dendritic Cell Motility in Tumors**

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Dendritic cells (DC) are cellular sentinels that gather tissue-derived antigens to coordinate adaptive immunity, including anti-tumor responses. DC residing in the tumor microenvironment can drive infiltration and activation of cytotoxic T cells. However, scant data exists on the temporal dynamics of DC influx into and out of tumors, as well as the cytoskeletal regulators driving their motility through tissues. Previous studies have reported opposing roles of the actin nucleators Arp2/3 and formins in promoting and restraining, respectively, the motility of immature DC ex vivo. We examined behaviors of tumor-resident DC using transplanted tumors and intravital imaging. Over several days of tumor outgrowth, we imaged lineage-marked tumor-resident DC or newly infiltrating immature DC within the same tumor transplant. Mature, tumor-resident DC were rounded and immobile, while their immature counterparts were elongated and migrated rapidly. DC in tumors that were cleared by the adaptive immune system meanwhile were reduced in number and remained motile over several days. To directly probe the cytoskeletal regulators of DC motility inside tumors, we imaged intact explants from progressively growing tumors and measured DC motility toward a chemoattractant. Consistent with
previous reports, inhibitors of myosin contractility or formin activity promoted DC motility, while inhibiting Arp2/3 did not. We further probed the roles of these cytoskeletal regulators on protrusion size, speed, and cell motility in DC migrating in confluent monolayers of tumor cells. These revealed fast motility of immature DC via rapid squeezing around tumor cells, in conjunction with rapid protrusion dynamics. Mature DC meanwhile were less well spread and appeared trapped in place by the surrounding tumor cells, mirroring tumor resident DC in explant imaging. Our observations reveal the origins and shifting behaviors of DC residing in tumors that overcome the host immune response. We propose that matured DC fail to exit the tumor, in part due to formin and myosin activity. We further probe DC motility through confluent tissues, revealing new shape and motility parameters enforced by confluent tissues.

P1357

Sars-cov-2 utilizes more robust mechanism to bind human ace2 than sars-cov
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The worldwide outbreak of COVID-19 caused by SARS-CoV-2 virus poses a serious threat to human health. SARS-CoV-2 virus begins to infect the human body through its spike (S) protein interacting with the angiotensin converting enzyme 2 (ACE2) of the host cell. Therefore, in order to develop treatments for COVID-19, it is urgent to understand the basic mechanism of how the SARS-CoV-2 S protein receptor binding domain (RBD) binds to ACE2. Here, we implemented a multi-scale calculation approach to study the binding mechanism of ACE2 and S proteins of SARS-CoV-2 as well as SARS-CoV. The electrostatic characteristics, electrostatic potential, electric field lines and electrostatic forces of SARS-CoV and SARS-CoV-2 are calculated and compared. The results show that both SARS-CoV and SARS-CoV-2 S proteins are attractive to ACE2 under the influences of electrostatic force. However, due to the mutations from SARS-CoV S protein to SARS-CoV-2 S protein, the residues of electrostatic characteristics are very different, and such differences were analyzed in detail. Compared with SARS-CoV, SARS-CoV-2 uses a more powerful strategy to bind with ACE2, which involves more salt bridges and hydrogen bonds in the binding interface than SARS-CoV. Besides, key residues involved in the salt bridges help SARS-CoV-2 S RBD to be attracted by ACE2 from a long distance. This study also identified key residues related to salt bridges and hydrogen bonds, which may be considered as drug targets to help future treatment designs against COVID-19.

P1358

An in-silico analysis of micrornas and transcription factors regulating IL-10 in acute respiratory distress syndrome
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Objectives: Antiviral immune responses initiated in the host cell leads to clearance of viral reservoirs from the infected cell. This excessive immune activation culminates into host cell damage. Interleukin-10 (IL-10), an anti-inflammatory master regulator, has a crucial role in regulating the excessive activation of pro-inflammatory cytokines in various acute respiratory infection, thus restricting pulmonary damage and development of ARDS. The study aimed at identification of the common regulating miRNAs of IL-10 and transcription factors (TF) affecting the expression of IL-10 by computational biology tools that can
play a significant role in ameliorating the development of pulmonary damage in COVID-19.

**Method:** Transcriptome related to the regulation of IL-10 were identified from TRRUST, RegNetwork, ENCODE, JASPAR and CHEA databases. Regulating miRNAs of IL-10 and its TF were identified and assorted from miRDB, miRBase, miRNet Version 2 and TargetScan. Functional enrichment, disease relationship, and KEGG pathway analysis of co-differentially regulated miRNAs of IL-10 and its TF were also identified by MIENTURNET MIEAA. **Result:** We identified four common miRNAs (hsa-miR-106a-5p, hsa-miR-24-3p, hsa-miR-204-5p, let-7c-5p) and two transcription factors (STAT3, IRF-1) having regulatory functions over IL-10. hsa-let-7c-5p and hsa-miR-106a-5p were found to regulate the TF STAT3 of IL-10 whereas hsa-let-7c-5p regulated TF IRF1 of IL-10. The role of IL-10 has been reported in SARS-CoV, SARS-CoV-2 and other respiratory viral infections. Although evidences regarding the role of the four miRs in respiratory viral infections are sparse, all have been shown to be upregulate viral diseases like chronic hepatitis B infection. The hsa-miR-24-3p was found to be upregulated in H5N1 avian influenza virus whereas hsa-miR-204-5p and hsa-let-7c-5p were associated with hepatitis C viral infection. **Conclusion:** hsa-miR-106a-5p, hsa-miR-24-3p, hsa-miR-204-5p, let-7c-5p were found to be associated with expression of IL-10 and its regulating TF in several viral infections. Further studies should be undertaken to elucidate the role of these miRs in respiratory viral infections and to assess the functional significance of these miRNAs in the larger perspective of pro-inflammatory mediated host cell damage. The identification of these miRNAs can help us understand the development of lung damage and ARDS in COVID-19 disease.

**P1359**

**Investigating the anti-inflammatory effect of curcumin and piperine in preventing biomaterial rejection in vitro**

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The inflammatory response to foreign biomaterials is a significant concern in various medical procedures. Chemokines and cytokines recruit monocyte-derived macrophages to the site of the implanted biomaterial, at which they adhere to and attempt isolate it from the body. This typically results in device failure and often requires replacement later in life. Natural products have garnered attention recently as potential therapies that could have anti-inflammatory properties. Curcumin, a yellow hydrophobic polyphenol extract from turmeric, has been shown to have anti-inflammatory characteristics. However, it has a poor bioavailability as it is quickly metabolized upon consumption. Piperine, an alkaloid component in black pepper, has been shown to increase the bioavailability of other nutrients. Through in vitro THP-1 cell adhesion assays, our data show that curcumin alone and in combination with piperine inhibit inflammatory cell attachment to polyurethane films, a common biomaterial. Ongoing experiments with an ex vivo Chandler Loop apparatus are being conducted to confirm the in vitro findings. These results indicate that curcumin and piperine have potential anti-inflammatory properties and warrant further investigation as potential therapies for biomaterial rejection.
**Determinants of Signaling Specificity for DBL-1/BMP in the Immune Response of the Nematode Caenorhabditis elegans**

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Innate immunity is the first line of defense against infection. It is a defense system composed of many levels of response, from physical barriers like the skin to release of antimicrobial peptides. When exposed to infection, the nematode C. elegans mounts an innate immune response through highly conserved cell signaling pathways to regulate the release of antimicrobial factors. BMP (bone morphogenetic protein) signaling, a type of TGF-β family signaling pathway involved in development and tissue homeostasis, is known to play a role in innate immunity. Our research aims to determine the way in which the C. elegans BMP like pathway - regulated by the ligand DBL-1 - interacts with other signaling pathways in order to confer immunity. The DBL-1 pathway plays a significant role in development and we are interested in discovering how it is able to differentiate a response specific to immunity, separate from its other functions in the worm. Through survival analysis we have shown that when exposed to pathogenic bacteria, expression of the DBL-1 effector sma-3 in either the hypodermis or pharynx is capable of improving survival compared to sma-3 mutants. We have also shown that mutation of the BMP like ligand TIG-2 results in similarly impaired survival rates as our dbl-1 mutants. These results suggest possible cross-talk between the pharynx and the intestinal site of infection, potentially through a non-canonical signaling pathway. Through the use of qRT-PCR we have found two immune-related genes with expression patterns that indicate regulation by DBL-1 signaling. We plan to use this data to further examine in which tissues DBL-1 signaling plays the largest role in the immune response of the worm and whether canonical or non-canonical DBL-1 activity is responsible for this function.

**LLPS in Genome Regulation**

**Condensation of the LINE-1 Retrotransposon Enables Dynamic Nucleic Acid Interactions**

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LINE-1 (L1), the major autonomous human retrotransposon, encodes two proteins: ORF1p, a structural protein, and ORF2p, a catalytic protein. ORF1p is necessary for L1 retrotransposition, but its role in the L1 life cycle is poorly understood. We observed that ORF1p phase-separated into droplets at nanomolar concentrations in *vitro*. Single-molecule DNA curtain assays revealed that ORF1p could recruit L1 RNA to dsDNA in condensates and could undergo linear diffusion along individual DNA double-helices. ORF1p also formed condensates in cells that were far less mobile in the nucleus than in the cytoplasm. One protein motif that may play a significant role in ORF1p condensation is the stammer motif, a three-residue insertion in ORF1p's coiled-coil trimerization domain. The stammer is thought to increase the flexibility of the coiled coil and facilitate *trans* interactions between ORF1p trimers. ORF1p stammer deletions and mutations strongly modulated both ORF1p condensate formation in cells and retrotransposition rate. We propose that ORF1p condensation helps orchestrate recruitment of the LINE-1 ribonucleoprotein to sites of retrotransposition in the genome.
P1362

**Surface condensation of a pioneer transcription factor on DNA**

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Recent work suggests that transcription factors and other proteins form transcriptional condensates via liquid-liquid phase separation. However, it is still unclear what physical mechanisms limit their size and whether phase separation is the best description of their physical nature. Here, we use optical tweezers to show that the transcription factor Klf4 on its own can form surface condensates on single DNA molecules at concentrations below those where Klf4 phase separation occurs in solution. We discover a switch-like transition from a thin layer to a thick condensed layer similar to prewetting in soft matter physics. Surface condensation limits the size of condensates, providing a mechanism by which DNA could control the size of transcriptional assemblies. Polymer surface mediated condensation reconciles several observations that were previously thought to be at odds with the idea of phase separation as an organizing principle in the nucleus.

P1363

**Liquid-Liquid Phase Separation Drives Compartmentalization and Protection of Telomeres**

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Telomeres are repetitive DNA sequences that safeguard chromosome ends in eukaryotes. The recruitment of shelterin components is essential for preventing undesired DNA damage repair (DDR) at telomeres and chromosome fusions, but the mechanism of telomere protection is not well understood. Here we show that shelterin components and telomeric DNA form liquid condensates in vitro, driven by heterotypic interactions between the TRF2 and TRF1 subunits and telomeric DNA. Phase separation in response to telomeric DNA is modulated by the N-terminal charged domains of TRF2 and TRF1. Protein and nucleic acid factors present in the condensate, as well as their stoichiometries, affect material properties. Telomere associated factors such as nucleosomes and TERRA (a noncoding RNA) are recruited to the shelterin condensates while the DDR factor RPA is not. These results suggest that biological condensates could regulate whether DNA damage factors can access the telomere, providing an explanation for how shelterin prevents the accumulation of DDR signals at chromosome ends.
Light-controlled 3D genome reorganization
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The three-dimensional structure of the human genome is important for maintaining proper gene expression and nuclear function. However, whether a locus’s position in the nucleus is a cause or consequence of its gene expression state is difficult to tease apart. Building on previous optogenetic technologies developed in the lab, we built a novel method for precise manipulation of locus repositioning on minute timescales using light. The technology utilizes a ‘seed’ protein that binds to the locus of interest and can nucleate liquid-like droplets of a phase-separating protein, here FUS-IDR, using the previously described Corelet system. When we locally activate by illuminating a small region of interest at the target locus that is bound by the seed protein, a FUS droplet forms and attaches to the locus of interest through surface tension. With specific activation patterns, we can encourage two locus-attached FUS droplets to merge together, creating one droplet with two loci attached to the periphery. Then, upon deactivation, the FUS droplet shrinks and the two loci follow the surface of the shrinking droplet to merge. This 20-minute activation/deactivation pattern can be utilized to bring together loci of interest across 1-micron sized spaces, or even repositioned to encourage interaction with specific nuclear structures like the lamina. Rapid repositioning of specific loci will allow for new understanding of the causal relationship between nuclear structure, gene expression and epigenetic reprogramming during differentiation, in addition to many other potential uses.

Inhibition of DEAD-Box RNA Helicase 3 attenuates stress granule assembly
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Stress granules (SGs) are non-membranous cytosolic protein-RNA aggregates that process mRNAs through stalled translation initiation in response to cellular stressors and in disease. DEAD-Box RNA helicase 3 (DDX3) is an active target of drug development for the treatment of viral infections, cancers, and neurodegenerative diseases. DDX3 plays a critical role in RNA metabolism, including SGs, but the role of DDX3 enzymatic activity in SG dynamics is not well understood. Here, we address this question by determining the effects of DDX3 inhibition on the dynamics of SG assembly and disassembly. We use two small molecule inhibitors of DDX3, RK33 and 16D, with distinct inhibitory mechanisms that target DDX3’s ATPase activity and RNA helicase site, respectively. We find that both DDX3 inhibitors reduce the assembly of SGs, with a more pronounced reduction from RK-33. In contrast, both compounds only marginally affect the disassembly of SGs. RNA-mediated knockdown of DDX3 caused a similar reduction in SG assembly and minimal effect on SG disassembly. Collectively, these results reveal that the enzymatic activity of DDX3 is required for the assembly of SGs and pharmacological inhibition of DDX3 could be relevant for the treatment of SG-dependent pathologies.
Mitophagy and Autophagy in Neurons

P1366

The role of post-translational modifications of cofilin in the regulation of mitophagy

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Cofilin is a small actin-binding protein that depolymerizes actin filaments. Cofilin was suggested to be involved in the regulation of mitochondrial functioning. Mitochondria perform multiple functions for the maintenance of cellular homoeostasis. The removal of damaged mitochondria through autophagy (mitophagy) is crucial to cellular physiology of the nerve cells. Defects in mitophagy are associated with the disturbance of tissue respiration. Molecular pathways involved in the regulation of mitophagy, and the role of post-translational modifications (PTMs) of proteins in this process remain poorly understood. The aim of this research was to study the role of PTMs of cofilin (phosphorylation and ubiquitylation) in mitophagy. Hippocampal cells were isolated from mouse embryos of C57BL/6 mice (E18). The primary culture of hippocampal cells was incubated with the proteasome inhibitor MG132 (5 μM). Confocal fluorescence microscopy and fluorescence-lifetime imaging microscopy were applied for the mitochondrial respiration study. Western blot and immunocytochemistry were used to analyze the expression of cofilin, ubiquitin (linkage-specific K63) and LC3B (an autophagy marker). Analysis of the NAD(P)H fluorescence lifetime showed a difference in the amplitude of the bound form of NAD(P)H in astrocytes and neurons, which indicates a different activity of oxidative phosphorylation and glycolysis in these cells. The co-localization of cofilin, phospho-cofilin, ubiquitin and LC3B with mitochondria shows the involvement of these proteins in the regulation of mitochondrial functions. The decrease in the activity of mitochondrial respiration were revealed in nerve cells under the action of the proteasome inhibitor. At the same time, MG132 led to the changes in the level of monomeric cofilin, phospho-cofilin and cofilin modified by K63-ubiquitin. The K63 ubiquitin chains are known to be involved in the degradation of ubiquitylated proteins by autophagy. LC3B expression was elevated in the nerve cells under MG132 treatment, indicating autophagy/mitophagy activation. Redistribution of phospho-cofilin, cofilin modified by K63-ubiquitin and LC3B between cytoplasm and mitochondria in the nerve cells under the action of the proteasome inhibitor shows the involvement of phosphorylated and ubiquitylated forms of cofilin in mitophagy. Thus, the role of PTMs of cofilin in regulation of mitophagy and mitochondrial respiration was shown for the first time. Understanding the molecular mechanisms involved in the regulation of PTMs of cofilin (phosphorylation and ubiquitylation) is essential for the diagnosis and treatment of the nervous system diseases. This work was supported by RSF (project # 17-75-10202) and RFBR (project # 18-34-00690).

P1367

Dynein Adaptor Protein RILP Controls mTOR-sensitive Neuronal Autophagy

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Microtubule-based molecular motors play critical roles in diverse cellular processes, including spatial organization and redistribution of organelles. The highly conserved retrograde motor, cytoplasmic dynein, is recruited to specific subcellular cargoes by a newly-emerging class of coiled-coil domain containing ‘adaptor proteins’. We have now discovered that dynein adaptor RILP, Rab-Interacting Lysosomal Protein, regulates the neuronal autophagy pathway through multiple sequential interactions with key autophagy proteins as well as dynein (Khobrekar et al., 2020). We find this RILP behavior to be controlled by mTOR kinase, a key regulator of autophagy. Defects in autophagy have been implicated in neurodegeneration and development, but how this pathway is organized and regulated in neurons remains poorly understood. We now show that RILP binds neuronal autophagosomes through three LC3-interacting regions (LIRs) and late endosomes through a non-overlapping Rab7-binding site. RILP is essential for AP and LE processive retrograde transport in axons. RILP depletion or mutations in its LC3-binding LIR motifs also decreases axonal AP number, identifying an unexpected RILP role in AP formation. During autophagosome formation, RILP directly interacts with ATG5 on growing phagophore membranes. Immunofluorescence and in vitro binding assays show that RILP interacts with ATG5 through the N-terminal dynein binding domain, thus precluding premature dynein recruitment and transport of incompletely formed phagophores. This novel competition between RILP N-terminal interactors ensures initiation of retrograde transport of only the fully-formed autophagosomes towards the degradative compartment. We also find that RILP expression is upregulated and the protein actively recruited to newly forming autophagosomes upon mTOR inhibition in neuronal and non-neuronal cells. Finally, RILP depletion and LIR motif mutations impede autophagic turnover of p62/Sequestosome-1 resulting in its cytoplasmic aggregation, a pathological feature often associated with a variety neurodegenerative diseases. Thus RILP, through its multiple interactors, integrates processes of autophagosome formation and retrograde transport to control autophagic turnover. These basic findings have important implications for understanding neuronal function, development, and disease.


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Aging represses autophagosome biogenesis but not autophagosome transport or maturation in neurons

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Autophagy defects are implicated in multiple late-onset neurodegenerative diseases. Since aging is the most common shared risk factor in neurodegeneration, we assessed multiple parameters of autophagy in mammalian neurons during aging. Using dorsal root ganglion neurons from mice of different ages, we previously determined that autophagosome biogenesis drastically decreases in neurons during aging. Despite this striking inhibition of biogenesis, we detected no age-related change in the number of autophagosomes present in the distal or proximal axon. We next assessed autophagosome transport and concomitant acidification and maturation. Using a tandem mCherry-GFP-LC3B marker to monitor autophagosome maturation, we identified an increase in the maturation of autophagosomes in the distal axon of neurons from aged mice. This change was specific to the distal axon, as we did not observe a change with age in the maturation of autophagosomes in the proximal axon. Interestingly, we did not did not detect gross, age-related changes in the transport of axonal autophagosomes. Focusing
on the autophagosomes moving retrograde in the distal axon, we observed no age-related changes in total run length, net displacement, pause duration, number of pauses, or average speed. We did identify age-related alterations in the proportion of autophagosomes that moved retrogradely, anterogradely, or bidirectionally in the distal axon. We observed that a higher proportion of immature, nonacidified autophagosomes moved anterograde with increasing age, while a higher proportion of mature, acidified autophagosomes moved retrograde in neurons from aged mice. Taken together, these data suggest that autophagosome formation in the distal axon is the predominant age-related change in the neuronal autophagy pathway, while autophagosome transport and maturation are relatively unaffected by age. Thus, therapeutic efforts to enhance autophagy in the context of aging or neurodegenerative disease should focus on the initial stages of the autophagy pathway.

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Age-related hippocampal memory loss induced by dysfunctional synaptic mitochondria accumulating tau PHF-1.
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Aging is a natural process that in the brain is manifested mainly by memory loss. Memory loss occurs, almost in part by synaptic failure and mitochondrial dysfunction in the hippocampus. In neurons, mitochondria are classified into synaptic and non-synaptic mitochondria accordingly to their subcellular localization. Interestingly, synaptic mitochondria are more susceptible to damage than non-synaptic mitochondria, but why this occurs is unknown. Also, the most common cause of damage in aged neurons is the accumulation of post-translational modified proteins, such as phosphorylated tau protein. Phosphorylated tau has been related to mitochondrial dysfunction; however, the exact mechanism by which tau promotes mitochondrial dysfunction is unclear. Interestingly, tau is reported to interact with several mitochondrial proteins, suggesting a direct link between tau and mitochondria. Therefore, we proposed that hippocampal memory loss could be induced by dysfunctional synaptic mitochondria due to the accumulation of tau PHF-1 (Ser396/404). To validate this hypothesis, we first evaluated the cognitive abilities of C57BL/6 mice at 3, 12 and 18 month-old (mo). We showed hippocampal memory loss since 12 mo, exacerbating it at 18 mo. This impairment is accompanied by mitochondrial dysfunction, shown as a decrease in ATP content and increased oxidative damage in the aged hippocampus. Interestingly, we showed that mitochondrial dysfunction is mainly mediated by synaptic mitochondria, which fail before and are more sensitive than non-synaptic mitochondria. In fact, we demonstrated that synaptic mitochondria undergo ATP production deficit, ROS overproduction, and calcium overload at 12 mo, while non-synaptic mitochondria fail at 18 mo. Thus, defects in ATP production of synaptic mitochondria correlate with hippocampal memory loss; suggesting that mitochondria at the synapses will be target of neurotoxic agents. Effectively, our results reveal that tau PHF-1 increase in the hippocampus at 12 and 18 mo. More importantly, we showed for the first time that tau PHF-1 accumulates inside hippocampal mitochondria of aged mice, demonstrated by immunogold electron microscopy and proteinase K assays. Moreover, we found that this PHF-1 tau accumulation occurs mainly inside the synaptic mitochondria, compared with non-synaptic mitochondria. Finally, we showed that tau PHF-1 induce dysfunction of healthy mitochondria; strongly suggesting that tau PHF-1 induce early failure of synaptic mitochondria at an advanced age. Altogether, these results suggest that tau PHF-1 accumulation results in the early synaptic mitochondrial
dysfunction, contributing to memory loss during aging and proposes a new potential therapeutic target to prevent age-associated cognitive decline.

New Techniques in Cell Biology: Algorithms

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Enhanced developmental defect screening via Deep Learning

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A developing tissue must evolve into a precise target shape to functions correctly. Embryonic tissues, however, do not always change shape properly, which results in fetal malformations. A challenge in developmental biology and maternal-fetal medicine is to pinpoint exactly when and why tissue morphogenesis begins to deviate. To analyze this question, researchers and doctors use imaging data.

The complexity and size of this imaging data has grown rapidly over the past decade. However, the sophistication of our methods for analyzing morphological mutations has not grown at the same rate. Many developmental biologists still qualitatively identify mutant organisms to identify when the anomaly first presents itself. To help solve this problem, we have developed a Deep Learning model to detect the appearance of an anomaly in video data of a developing tissue. We employ a machine learning framework to analyze when and where anomalies first appear in developing mutant embryos.

Using a pipeline that includes transfer learning, PCA, and an unsupervised random forest algorithm, we have created a model that can distinguish between images of mutant and wild-type fruit fly (*Drosophila melanogaster*) embryos. Our model can also cluster images based on the type of mutation present. Identifying at what developmental time point mutants begin to cluster apart from WT can pinpoint morphogenetic dysregulation. Additionally, identifying which mutants cluster together can deliver fundamental biological information of how certain proteins work together to control similar elements of shape change. Our model only requires dozens of movies and does not require high compute resources making it a transferable pipeline for diverse developmental contexts. In conclusion, our model demonstrates that machine learning algorithms can be effectively applied to the problem of anomaly detection in developing organisms with potential clinical application. This model also represents a novel application of machine learning to developmental biology, demonstrating the vast array of possible applications of machine learning to the field.

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Intelligent connectomic analysis tool for dense neuronal circuits

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Advancements in microscopy techniques, labeling methods, preparation procedures, data analysis informatics and computing infrastructures have made high-resolution imaging and visualization of large tissue blocks with subcellular resolution using electron microscopy (EM) a possibility. Armed by these advancements, long-standing neurobiological questions regarding structure-function relation are being extensively studied through elucidating and quantifying detailed neural networks. However, the reconstruction and analyses of neuronal networks remain challenging in part due to the lack of readily
available tools for large connectomic data analysis. We are developing a deep learning (based on a customized 3D U-Net) powered intelligent connectomic analysis (ICA) tool that could rapidly perform boundary segmentation, cell reconstruction and synaptic connections from EM volumes to answer biological questions that are currently untraceable. The tools could also enable investigators to correlate structural with functional connectivity. We proved the performance of an alpha version of the ICA tool by confidence and verification tests and confirmed that the tool can accurately detect and trace neurons on EM images and can map, register and correlate between EM and LM images. Using a testing dataset acquired from mouse retina blinded to truth, we achieved the following performance metrics:

1. Boundary detection accuracy: Vrand (Rand Score) = 0.979, Vinfo (Information Theoretic Score) = 0.988
2. Neuron tracing: ARE (Adapted Rand Error) = 0.09
3. Correlative transformation: F1 score = 0.82

We will continue improving the ICA tool and expand training and testing data to include additional regions of the nervous system and model systems.

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Active learning enabled intelligent annotation for deep model training applied to EM connectomics analyses
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Active learning is a machine learning approach where a learning algorithm can interactively query a user to label uncertain data for ground truth (GT) annotation. The approach could be beneficial in applications where unlabeled data is abundant but the labeling of GT is expensive. We applied a computer-directed active learning approach for intelligent annotation to train a deep convolutional neural network (CNN) model for the analysis of electron microscopy (EM) connectomics data. For active learning evaluation, we started with 10% of the GT for training the CNN and added an additional 10% GT by computer-directed active learning selection (total 20% of GT) and evaluated the achievable neuronal boundary detection accuracy with the 5 times reduction of GT. To enable active learning, a 3D U-Net architecture was extended by inserting dropout layers before each down-sampling layer and after each up-sampling layer. During network applications, 8 predictions were performed for each input image by random dropouts. The standard deviations of the 8 predictions are calculated as the prediction uncertainty values. The active learning training procedure consists of the following steps: 1. Train extended 3D U-net by randomly selected 10% of GT objects; 2. Calculate the prediction uncertainty map for each training image by Monte Carlo dropouts; 3. Select additional 10% of GT objects with high uncertainty values; 4. Perform transfer learning using 20% of GT objects with the CNN from step 1. as the base model. The neuronal boundary detection accuracy metrics Vrand (Rand Score) and Vinfo (Information Theoretic Score) are used for performance evaluation. We found the accuracy results from active learning (using 20% GT) outperform the training using 100% GT. This indicates that the training by a properly selected subset of GT could generate a better network than when training with a larger amount of undirected GT. This result is significant because the active learning approach could reduce 80% of the expensive GT creation process and yielded improved results. We will further validate our active learning approach by applying it to new datasets for efficient and guided annotation.
A deep-learning based approach for integrated multi-level phenotyping of cell edge dynamics from live-cell imaging reveals heterogeneity in drug responses at the single-cell level
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Cell movement plays a fundamentally critical role both in physiological and pathological processes. It is a complex process exhibiting significant heterogeneity at each different level of organization in biology and across different time scales. Due to this heterogeneity, population-level drug characterization conceals a substantial amount of information regarding individual cellular responses. Therefore, a single-cell-based quantitative assay using live-cell imaging is required to measure the drug effects on cell motility accurately. We took a modular approach by first identifying the subcellular phenotypes using the unsupervised learning framework we developed, DeepHACKS (Deep phenotyping of Heterogeneous Activities in the Coordination of cytoskeleton at the Subcellular level). DeepHACKS divides the leading edge of a cell into small areas (windows) and identifies distinct patterns from the acquired velocity time series from each window by integrating LSTM (Long-Short Term Memory) autoencoders with the prior information from traditional machine learning outcomes from our previous study. Using this framework, we identified rare subcellular phenotypes, such as ‘accelerating’ and ‘bursting’ protrusion phenotypes. By connecting the local protein dynamics with subcellular phenotypes, we revealed that the accelerating protrusion phenotype is mediated by unconventional temporal coordination of Arp2/3 and VASP, suggesting that fine temporal control in protein dynamics is responsible for the differential subcellular response. Then, we performed cell phenotyping based on the distribution of subcellular phenotypes per cell, revealing a specific population of cells exhibiting both high proportions of ‘accelerating’ and ‘bursting’ protrusion phenotypes. Morphodynamic features of these cells suggest possible spatiotemporal coordination between the two subcellular phenotypes. Furthermore, we applied DeepHACKS to a single-cell based drug exchange assay, tracking the motility of the same cell before and after the drug treatment while keeping the spatial context of cell-cell contact within the colony. We further subdivided subcellular phenotypes into fine-grained phenotypes using machine learning. Our result revealed that each cell is assigned to ‘susceptible’ or ‘resistant’ groups against Cytochalasin D depending on the level of changes in the fine-grained ‘accelerating’ and ‘bursting’ protrusion phenotypes at the single-cell level. By combining the molecular and spatial context of a population of cells, our machine learning platform has the potential to elucidate the mechanism where a specific population of cells is more drug-resistant than others from live-cell imaging.

AI Microscopy: deep learning minimizes the impact of fundamental microscopy limitations
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Fluorescence microscopy has contributed to numerous major discoveries in life sciences, despite the limitations shared by all imaging systems. Typically, fluorescence microscopes excel at one of three core factors that modulate image quality and/or sample viability: spatial resolution, temporal resolution, and signal-to-noise ratio. Optimizing one of these factors often comes at the detriment of the others, inherently limiting fluorescence microscopy. To minimize the impact of these limitations, deep learning (DL) enabled microscopy image restoration is starting to be adopted [1,2]. Here we illustrate how customized Residual Channel Attention Network (RCAN) [3] can be used for restoring images on multiple imaging modalities. DL restoration reduces light exposure allowing prolonged cell viability in long time lapse image sequences. Using the hybrid cloud-desktop platform, Aivia / Aivia Cloud, we trained and applied a customized RCAN to restore EGFP-labeled mitochondria time lapses taken at reduced light exposure (0.2 mW). With this approach, we extended the imaging period from 50 to 2,600 volumes, acquired every 3 s on an instant structured illumination microscope (iSIM) with no detectable photobleaching. The restored images showed ample spatial resolution to segment individual mitochondria in a semi-automatic way. Using the approach described above, we significantly shortened the time needed (3x reduction compared to imaging ground truth) to image samples on a resonant-scanning confocal system. Additionally, we explored the use of RCAN to enhance the spatial resolution of standard confocal images to that of STED microscopy. Whereas microtubule and nuclear pore complexes appeared resolution-limited on the confocal image, decorrelation analysis showed that RCAN achieved 2x resolution improvement - similar to that of STED. We applied the approach to SiR-DNA labeled nuclei in live cells, imaged on a resonant-scanning confocal system. RCAN resolved chromosomal structures within the nuclei that were poorly defined in the raw image. In this work, we demonstrated that our modified RCAN can reduce the light exposure to extend live imaging duration while preserving spatial resolution for analysis; and enhancing spatial resolution of ordinary light microscopy to that of super-resolution microscopy. Additionally, we will discuss how our approach can be adapted to various imaging systems to overcome their specific limitations. 1. Weigert, et al., Content-aware image restoration: pushing the limits of fluorescence microscopy. Nat Methods, 15 (2018)2. Sasaki, et al., Deep learning enables long term gentle super resolution imaging. Poster. ASCB-EMBO 2018.3. Zhang, et al., Image super-resolution using very deep residual channel attention networks. ECCV 2018.

Towards a Machine-Learning-Assisted Dielectric Sensing Platform for Point-of-Care Wound Monitoring
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Currently, an estimated 6.5 million patients in the US each year experience a wound that fails to heal. Chronic wound management would be revolutionized if doctors could monitor wound healing in real time remotely and predict accurately which wounds will heal and which will not. This study is conducted to identify pH and dielectric constant in wounds of mouse model, and its relationship to the healing process. We measured pH, and both real and imaginary part of dielectric constants in wounds with different conditions, including splinted, non-splinted and UV-burnt treatments. Through a machine learning algorithm and principal components analysis, our approach could reproducibly distinguish
wounded from non-wounded skin. The experiments also defined the range that will be used to develop miniaturized sensors that can be implanted in wounds.

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**SpinX: Time-resolved 3D Analysis of Spindle Dynamics using Deep Learning Techniques**

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Live-cell movies generate terabytes of data. However, manual analysis of this data is prone to error and can easily exhaust days of research time, thus limiting the insights that can be gleaned from cutting edge microscopes. Automated analysis has been hard because of discontinuities between the distinct frames of 3D live-cell movies. We present SpinX, a comprehensive and extensible computational framework which bridges the gaps between discontinuous frames in time lapse movies by utilising state-of-the-art Deep Learning technologies and modelling for 3D reconstruction of highly mobile subcellular structures. Using SpinX, we are now in a position to precisely track and analyse the movements of multiple subcellular structures within minutes, including the cell cortex, chromosomes and the mitotic spindle. We demonstrate the utility of SpinX by employing it to define the precise roles of spindle movement regulators that ultimately determine the plane of cell division. We illustrate the extensibility of SpinX by showing how it can also be used to infer the regulation of complex cortex-microtubule interactions. Our analyses reveal previously unrecognised roles for the evolutionarily conserved Dynein motor and MARK2/Par1 polarity kinase in regulating the 3D movements of the mitotic spindle. Thus, SpinX provides an exciting opportunity to study spindle dynamics in relation to the cell cortex using hundreds of time-resolved 3D movies in a novel way.

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**4D Cell Biology**

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New methods in stem cell 3D organoid tissue culture, advanced imaging, and big data image analytics now allow tissue-scale 4D cell biology but currently available analytical pipelines are inadequate for handing and analyzing the resulting gigabytes and terabytes of high-content imaging data. We expressed fluorescent protein fusions of clathrin and dynamin2 at endogenous levels in genome-edited human embryonic stem cells, which were differentiated into intestinal epithelial organoids. Lattice light-sheet imaging with adaptive optics (AO-LLSM) allowed us to image large volumes of these organoids (70 × 60 × 40 μm xyz) at 5.7 s/frame. We developed an open-source data analysis package termed pyLattice to process the resulting large (~60 GB) movie data sets and to track clathrin-mediated endocytosis (CME) events. We then expressed fluorescent protein fusions of actin and tubulin in genome-edited induced human pluripotent stem cells, which were differentiated into human cortical organoids. Using the AO-LLSM mode on the new MOSAIC (Multimodal Optical Scope with Adaptive Imaging Correction) allowed us to image neuronal migration deep in the organoid. We augmented pyLattice with a deep learning module and used it to process the brain organoid data.
Unraveling Nanotopography of Cell Surface Receptors
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Cells communicate with the environment via surface receptors, but nanoscopic organisation of these receptors with respect to complex cell surface morphology remains unclear. This is mainly due to a lack of accessible, robust and high-resolution 3D imaging methods. We have developed a user-friendly approach for mapping the topography of receptors at the cell surface with nanometre precision. The method involves coating glass coverslips with glycine, which preserves the fine membrane morphology while allowing immobilised cells to be positioned close to the optical surface for single-molecule fluorescence microscopy. Glycine coating of coverslips also prevents non-specific activation of cells as observed for poly-L-lysine coating. This is often accompanied by increased cell death. Cells on glycine-coated coverslips exhibit excellent viability. We also generated an advanced and simplified algorithm for the analysis of single-molecule localisation data acquired in a biplane detection scheme. These advancements enable direct and quantitative mapping of protein distribution on nano-morphology of plasma membranes with near isotropic 3D nanometre resolution. As demonstrated successfully for CD4 microclusters and segregation of CD45 from signalling receptors, the described workflow is straightforward quantitative technique to study molecules and their interactions at the complex surface nanomorphology of differentiated metazoan cells.

Oncogenes and Tumor Suppressors: Mechanisms of Disease

Induction of apically mistrafficked EREG disrupts epithelial polarity via aberrant EGFR signaling
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Disruption of the tyrosine-based YXXΦ basolateral trafficking motif (Y156A) in the EGF receptor (EGFR) ligand, epiregulin (EREG), in MDCK cells results in its apical mistrafficking, altered epithelial polarity, and transformation in vivo. However, the mechanism(s) underlying these effects are unknown. Using a doxycycline-inducible system, we now show that Y156A EREG induction in fully formed MDCK cysts in Matrigel results in direct and complete delivery of mutant EREG to the apical cell surface and formation of ectopic lumens that correlates with the in vivo transformed phenotype. These ectopic lumens form de novo rather than budding from the central lumen and depend on metalloprotease cleavage of EREG and EGFR activity. Moreover, the most common EREG mutation in human cancer (R147stop) results in its apical mistrafficking in engineered MDCK cells. Thus, apical mistrafficking of EREG results in a gain-of-function phenotype, rather than loss-of-function phenotypes commonly observed with mistrafficking of proteins.
Hormones as Effective Regulators of Short SERPINA1 Transcripts Encoding Alpha1-antitrypsin Bioactive Peptides in Vitro
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Alpha1-antitrypsin (AAT) is the most abundant SERine Proteinase INhibitor (serpin) in human plasma. C-terminal domain of the protein encoded by SERPINA1 gene exon 5 is the precursor of biologically active peptides, which have been found in humans in pathological states [Cercek L & Cercek B, 1992; Chang WC et al., 2008; Zhou J et al., 2010; Dichtl et al., 2000; Blaurock N. et al., 2016] as well as in normal condition (nNIF - neonatal NET-inhibitory factor) [Yost, 2016]. Previously we identified endogenous short SERPINA1 transcripts in hepatocellular carcinoma cell line HepG2. These transcripts may potentially contribute to the production of the active peptides along with the established proteolytic pathway. Here we show that in HepG2 cells steroid hormones and prolactin affect short transcripts expression, while the impact on the synthesis of the long transcripts is much less prominent. Prolactin, 17β-estradiol, and cortisol appear to be the down-regulators with maximum 4-, 2,2- and 4,4-fold inhibitory activity, respectively, at 10(-8)M. In contrast, testosterone and progesterone upregulate the expression of the short transcripts with maximum 3,2- and 3-fold elevation at 10(-7)M and 10(-6)M, respectively. Because hepatocellular carcinoma is more common in men than in women and considering that peptides act in favor of the tumor, our results may partially explain such a disparity in sex predisposition. Moreover, our results suggest that mother’s hormones, such as progesterone, may potentially drive nNIF production and may explain why NET-inhibitory peptides level rapidly decreases in the circulation of the infant shortly after delivery [Yost, 2016].

Analysis of the tumor suppressor gene CYLD expression in clear cell renal carcinoma
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IntroductionThe tumor suppressor gene Cyld has been of particular interest recently, due to its involvement in many forms of neoplasia including melanoma, hepatocellular carcinoma, colorectal adenocarcinoma, breast, head and cervical cancer and more recently in pancreatic cancer. The protein CYLD is a cytoplasmic enzyme with deubiquitinase properties. CYLD selectively hydrolyzes polyubiquitin chains, which are not involved in the degradation of proteins through the proteasome, but act as
scaffolds to form signalling regulatory complexes. The protein CYLD is involved in the regulation of signalling pathways associated with cell division, cell survival and the human immune system. Studies on the molecular function of CYLD have shown that it interferes with NF-kappaB, JNK, p38 and Wnt signaling. **Objectives** The present study aims to investigate the hypothesis that downregulation of the tumor suppressor protein CYLD is implicated in oncogenesis of clear cells, in renal clear cell carcinoma (ccRCC), by possibly regulating the ubiquitination of proteins that are involved in growth and survival. **Materials and Methods** Paraffin samples from 21 patients with ccRCC were used for immunohistochemistry experiments and RNA isolation. CYLD expression was determined using immunohistochemistry experiments, RNA isolation, reverse transcription and real time PCR. Ethical Committee approval has been obtained. In addition, cell culture techniques were applied to test the viability of cells that were expressing a wt or a mutant CYLD (CYLDC601S), which is catalytically inactive. For this purpose, transfection experiments were performed, followed by a clonogenic assay. **Results** Using immunohistochemistry experiments, preliminary results showed that CYLD expression is completely suppressed in the tumor tissue in 10/21 samples, whereas high CYLD expression is detected in adjacent physiological cells, as determined by immunohistochemistry. Preliminary data also showed that CYLD mRNA is underexpressed, approximately 40% in cancerous tissue, compared to the adjacent physiological tissue, as it is shown after isolation of RNA from paraffin samples and performing Real Time PCR experiments. Preliminary results of transfection experiments in 293FT cells and clonogenic assays showed that cell viability is higher when the catalytically inactive form of CYLD was expressed. **Conclusion** The results of the present study support the notion that CYLD has a tumor suppressing role in clear cell renal carcinoma and it inhibits proliferation in vitro, suggesting that it can be used in the future for prognostic and predictive purposes and potentially the development of targeted therapies.

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**Investigating the effect of Eya3 knockdown in zebrafish on innate immune cells**

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Rhabdomyosarcoma (RMS) is a malignant cancer that accounts for approximately half of pediatric soft tissue sarcoma cases (1). Decades of research on RMS improved understanding of different types of RMS and improved RMS treatment options. However, the overall survival rate of metastatic RMS patients remains poor (2). Current pre-clinical studies are being conducted to find potential advanced treatments, which could include immune-targeted therapies (3). The immune system plays a complex dual role in tumor progression; immune cells can either target tumor cells for destruction or promote tumor growth and spread. EYA proteins (EYA 1, EYA 2, EYA 3, EYA 4) are of interest in this dual role as EYA proteins have been noted to either promote or prevent immune responses depending on the context. Both mammalian EYA 4 and Drosophila EYA were discovered to promote an innate immune response against DNA, while mammalian EYA 3 showed suppression of tumor-associated adaptive immunity (4,5,6). Such results suggest that further research on determining roles of EYA in immune responses will be necessary. This study will be focused on assessing innate immune cell (both neutrophil and macrophage) responses to EYA knockdown. Morpholino injection will be conducted to knockdown EYA 3 in zebrafish and anti-L-plastin will be used for immunofluorescence which can stain both macrophages and neutrophils. Results for this study are yet to be determined. Findings of this study will be helpful in clarifying the intricate role of EYA proteins regarding innate immunity. This information will inform future studies aimed at developing immune-targeted therapies for RMS.
Potential targets for cancer therapy, and their role in cancer remains unclear. The goal of this research project is to elucidate the biochemical function of the expression of XAGE genes and their protein products in normal and diseased states. We will accomplish this goal by first generating an atlas of cancer cell lines that display XAGE expression. Then we will perform gain and loss of function studies to assess role of XAGE expression in cancerous phenotype. We will determine what pathways are perturbed in the presence and absence of XAGE expression by identifying binding partners of these proteins by IP-Mass Spectrometry. Based on previous studies of CTAs it is likely that XAGE expression and function is modulated through epigenetic events. In summary, our studies will shed light on the biochemical functions of the enigmatic and aberrant expression of XAGE proteins in normal and diseased states.

Uncovering MAGE-B2 function using unbiased proteomics and interactome analysis.

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Melanoma antigen genes (MAGE) are a group of tumor specific genes that are normally expressed in male germline cells and aberrantly expressed in a wide range of cancers. Cancers that express MAGE-B2, a member of the MAGE family have been shown to correlate with poor patient prognosis. Studies highlight MAGE-B2 to have high tumor inducing potential in mouse xenograft studies. Interestingly MAGE-B2 depletion in cancer cell lines by shRNA mediated knockdown decreases cell viability and decreases proliferation. Additionally, expression of MAGE-B2 in normal cells increases cell viability pointing to a marked pro-proliferative effect. The functional basis for MAGE-B2’s tumorigenic, pro-proliferative effect is unclear and has been sparsely investigated. We are seeking to establish a mechanistic basis for MAGE-B2 function. We propose to use MAGE-B2 as a prototypic member to understand the biochemical role of the MAGE family of proteins. To this end we designed recombinant expression constructs of MAGE-B2 with single and tandem affinity tags for generation of heterologous cell lines. We used the cells lines with robust expression of MAGE-B2 to perform unbiased proteomics and generate a global protein interactome for MAGE-B2. Our list of MAGE-B2 interacting proteins is highly enriched in RNA binding proteins (e.g. KHSRP, ELAV1 and AUF1) and enzymes involved in protein turnover (DCUN1D5, UBE2NL and PCMT1). We are currently validating binding partners by using reductionist approaches to probe binding to MAGE-B2. First, we will use a coupled invitro transcription and translation assay to enable rapid pairwise screening of interactors. We have generated expression clones of several of the MAGE-B2 interacting proteins in a pCS2-based plasmid bearing an SP6 promoter.
This expression system is particularly suited for synthesizing mRNA and protein in a coupled *invitro* transcription and translation system which represents an easy way for probing protein-protein interactions. Identifying robust protein binding partners for MAGE-B2 is key to ascribing a cellular function not only for this particular MAGE but also to the general MAGE family. We will then test validated binding partners for contribution to MAGE-B2’s established tumorigenic, pro-proliferative effect by siRNA mediated transcript knockdown. A successful validation will require at least partial recapitulation of the MAGE-B2 depletion effects in cancer cells. Using this systematic approach, we aim to outline a mechanistic basis for MAGE-B2 function.

**P1385**

**Biophysical investigation of p53(Y220C) mutant and its interaction with potent small molecule modulators**

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Transcription factor p53 is an oncosuppressor protein that is activated in response to various types of cellular stress. In many cases p53 regulates the expression of genes whose protein products lead to cell cycle arrest and/or apoptosis. In addition, in about 50% of human cancers p53 tumor suppressor inactivation occurs as a result of point mutations, primarily in the DNA-binding domain. Oncogenic mutation Y220C is one of the most common for p53 and is detected annually in about ~100,000 diagnosed cancer cases. The presence of this mutation violates the tertiary structure of the DNA-binding domain p53, which leads to destabilization of the protein, its partial denaturation and loss of activity. Stabilization of the mutant p53(Y220C) structure and activation of its disturbed transcription functions is possible with the help of small molecules described in the literature as stabilizers, activators or modulators. The aim of this project is to study the interaction of derivatives of MB725, developed in collaboration with the team of Prof. Matthias Baud (University of Southampton, UK) with recombinant p53(wt) and p53(Y220C). Protein expression was carried out in the bacterial strain *E.coli* BL21(DE3)pLysS. Purification of protein was performed by metal-chelate affinity, ion-exchange and gel-filtration chromatography. Binding of the compounds to proteins was subsequently assessed biophysically using differential scanning fluorimetry (DSF), surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC). Compounds showing high affinity for the mutant protein were selected for structural biology analysis by X-ray diffraction. At the moment, the optimization of conditions for growing crystals of the recombinant protein p53(Y220C) is being carried out. High quality crystals will be soaked with the most potent compounds. Diffraction data will be collected using Rigaku XtaLAB Synergy-S diffractometer with HyPix-6000NE hybrid photon counting detector. Further studies of the interaction of MB725 derivatives with the recombinant proteins p53(wt) and p53(Y220C) are important for the development of novel personalized anticancer drugs that target mutant proteins. The study was funded by RSF grant 19-74-10022 to EB. References Chasov V, Mirkayazova R, Zmeievskaya E, Khadiullina R, Valiullina A, Stephenson Clarke JR, Rizvanov A, Baud MG, Bulatov E. Key players in the mutant p53 team: small molecules, gene editing, immunotherapy. Frontiers in Oncology. 2020; 10. DOI: 10.3389/fonc.2020.01460
Genome-Wide CRISPR Screen Identifies Non-Canonical NF-κB Signaling as a Potent Regulator of Epithelial Homeostasis

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Epithelial cells divide during tissue development and after tissue stretching or wounding, but they arrest when a certain homeostatic cell density is achieved. This mechanism prevents tissue overgrowth and is known to be lost in cancer. Despite its importance, this mechanism is not fully understood. To find novel factors controlling homeostatic cell density, we performed a genome-wide CRISPR-Cas9 knockout (KO) screen on the EpH4 cell line. This is mammary epithelial cell line that retained normal epithelial features including homeostatic cell density control (these cells arrest at day 4 after reaching confluence). We transduced the cells with the GeCKO whole-genome gRNA library and selected by FACS for cells that continue to cycle at day 4 after reaching confluence. We sequenced the gRNAs and analyzed which genes were enriched in comparison to control. We tested three of the top enriched genes, which are Nf2 (a known tumor suppressor and regulator of Hippo signaling), Traf3 (TNF Receptor Associated Factor 3, a negative regulator of proliferation in blood cells), and Ubc12 (ubiquitin-like protein NEDD8-conjugating E2 ligase). We confirmed that KO of these proteins indeed led to overproliferation and multilayering at high density but not at low density. Then we focused on studying Traf3-dependent mechanisms of cell density-dependent proliferation. We found that the KO of non-canonical NF-κB pathway downstream kinase NIK or the pathway effector p100 reduces overproliferation in Traf3 KO cells, which suggests that this pathway is necessary for proliferation upon Traf3 loss. We also found by RNA-seq analysis that loss of Traf3 results in induction of innate immune pathways, including antigen presentation and interferon response pathways that could be partially restored by the p100 KO. We found that Traf3 KO cells overproliferate independently of Hippo signaling and cyclin kinase inhibitors, over-riding these classical density dependent proliferation control mechanisms. Our novel KO screen revealed both a known density-dependent proliferation regulator, Nf2, and two new regulators, Traf3 and Ubc12. We also found an unanticipated involvement of non-canonical NF-KB signaling in control of epithelial cell proliferation at high density. These studies extend our knowledge of cell density-dependent proliferation in epithelia, which is important for tissue maintenance.

Metabolism Shift of Epithelial Mitochondria Transplantation in Breast Cancer Cells Based on Phasor FLIM

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Mitochondria transplantation, either natural or artificial, has been an important topic to study, especially its potential ability to help dysfunction cells regain health. Recent studies have shown that while mitochondria in fueling the energy need for cell function, the influence of healthy mitochondria transplanted in cancer cells is seem to alter energy metabolism. These assays normally reflect data from bulk measurements, neglecting individual recipient cell characteristics. Whether single cell mitochondrial uptake influences longitudinal and real-time cellular metabolism, the difference of these assay readings remain unknown. We therefore are combining the use of the phasor approach to fluorescence lifetime...
imaging microscopy (FLIM) to bridge the gap of interest. NADH, a key co-factor involved in glycolysis and oxidative phosphorylation (OXPHOS) exists in either the free or bound form. The relative fraction of endogenous free and bound NADH is a useful method for monitoring changes in energy metabolism since the NADH/NAD+ pair is crucial for electron transfer through the mitochondrial electron transport chain. The phasor approach to FLIM analysis provides label-free, fit-free and sensitive method to identify different metabolic states of cells. To target specific recipient cells, we transfected mitochondria (COX8GFP) only in epithelial cells and stable cancer cell (mCherry) for recipient role. Our results have shown that the phasor distribution of breast cancer cells uptaken mitochondria from epithelial cells performed a left shift on the universal semi-circle phasor plot. This indicates recipient breast cancer cells metabolism has shifted from glycolysis to OXPHOS. Also, by averaging lifetime cursors on our FLIM phasor plot, we were able to confirm exogenous mitochondria location and which they have contributed to bound NADH lifetime. Our findings support that transferred mitochondria maintains its regular function thus bringing potential ability to induce cell apoptosis. The significance of this work demonstrates metabolism quantification of single cell scale using FLIM to show a clear shift towards OXPHOS. Quantification of the shift will later on be compared with golden standards methods such as seahorse assay. Our future work will be focused on the fate of mitochondria transplanted cancer cells using single cell RNA sequencing to reveal inner changes of the mitochondria signaling.

P1388

The role of antioxidant enzyme GSTA2 in the nucleolus of mutant fallopian tube epithelial cells

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High grade serous carcinoma (HGSC) is the most common ovarian cancer subtype and is characterized by a highly aggressive and genomically unstable phenotype. In a previous publication, we showed higher levels of GST (glutathione-S-transferases) isoenzymes in the fimbrial end of the fallopian tube relative to the ampulla. We suggest an upregulation of detoxification genes at an area normally bathed with cytotoxic factors is protected by GST’s that decrease the level of oxidative stress by conjugating glutathione (Y-L-glutamyl-L cysteinyl-glycine, GSH) to carcinogens and chemotherapeutic drugs. We used patient derived fallopian tube epithelial (FTE) cells incubated with 50μM H2O2 for 10 mins at room temperature to induce ROS, which was measured by fluorescence intensity change. Total γ-H2Ax (DNA damage marker) positive and negative FTE cells were expressed as a percentage of the total cell count for treated and untreated cells. A comparison of FTE tissues showed increased GSTA2 positive cells in the fimbria compared to the ampulla. There were significantly more GSTA2+ cells in the distal end of the fallopian tube (the high risk zone) compared to the proximal end. We probed FTE tissues for other GSTs and determined that GSTA 1-4 localized to different cellular compartments. GSTA2 localized within nucleophosmin positive compartments in the nucleus (nucleolus). FTE stably expressing mutant p53, which recapitulates early lesions of ovarian cancer, had higher basal levels of γ-H2Ax. γ-H2Ax localizes with GSTA2 in both nucleolus and non-nucleolus compartments of the nucleus. p53 modulates the activity of the RNA polymerases I and III. We observed that UBF, a nucleolar transcription factor, and GSTA2 co-localize in the nucleolus at the site of rRNA synthesis suggesting a role for GSTA2 in ribosomal biogenesis. FTE mutant p53 showed decreased co-localization of UBF and GSTA2 in the nucleolus. Our results suggest a role for GSTA2 in mediating the DNA damage response in fallopian tube epithelial cells. GSTA2 functions in the nucleolus to regulate rRNA synthesis therefore modulating ribosomal biogenesis.
and the cell’s ability to synthesize downstream antioxidants. The differential gene expression of GSTA2 in HGSC and fallopian tube tissues suggests a role in regulating the pathogenesis of ovarian cancer through detoxification mechanisms.

Other Kinesins

P1389

Controlling the activity of cargo-bound microtubule motors using optogenetics

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To dissect the roles of motor proteins in cargo transport, we developed optogenetic tools to transiently inhibit specific kinesin motors. Conventional approaches to inhibit motor proteins, such as siRNA, chemical-genetic inhibition, and dominant-negative expression, lack temporal control over regulation and are often irreversible. To overcome these limitations, we developed a tool to study the role of motor proteins in cargo transport with precise temporal control and with minimal perturbation to cells. We used the LOVTRAP optogenetic system that comprises of two components, the membrane bound Z-dark protein and LOV protein, the photo-responsive element. Upon blue light illumination, the protein of interest is released in the cytoplasm and in the absence of blue light, it re-binds the membrane-tethered component. To achieve reversible inactivation of motor proteins, we used the native autoinhibitory segments of different kinesins. These inhibitory segments help motor proteins remain inactive in the absence of cargo in a cell. Inhibitory peptides for kinesin-1, 2 and 3 were cloned onto the LOVTRAP system and these optogenetic inhibitors were then transfected into COS-7 cells. The processivity of lysosomes decreased when kinesin-1 or kinesin-2 was inhibited by the optogenetic inhibitor upon blue light illumination. Moreover, there was a stronger decrease when both kinesin-1 and kinesin-2 were simultaneously inhibited. Motility of early endosomes showed a similar trend upon inhibition of kinesin motors whereby their motility became less processive and more stationary. These tools have demonstrated successful inhibition of motor proteins in live cells with an additional layer of reversible control over their activity. Next, we will use these specific controllable inhibitors to study misregulation of motors and its impact on neurodegenerative diseases.

P1390

Determining the role of ZEN-4/KIF23 in C. elegans vulva development

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The Epidermal Growth Factor Receptor (EGFR) promotes numerous metabolic pathways critical for cancer cell proliferation [1], and EGFR signaling overactivation results in many types of epithelial cancers [2]. Vulva development in the nematode Caenorhabditis elegans is a superb model for studying LET-23/EGFR signaling regulation. Basolateral localization of LET-23/EGFR in the vulva precursor cells (VPCs) is necessary for signal activation and vulval cell fate induction. Underactivation or overactivation of LET-
POSTER ABSTRACTS

23/EGFR signaling leads to a vulvaless or multivulva phenotype, respectively. Therefore, dysregulations of vulva induction caused by gene mutations are powerful templates for epistasis analyses which position those genes within the LET-23/EGFR signaling pathway. One such potential gene is that of the kinesin-like protein ZEN-4, an evolutionarily conserved component of the centralspindlin complex involved in midzone organization during cytokinesis [3]. ZEN-4 also regulates arcade cell polarity in early morphogenesis of the pharynx [4, 5]. We previously found that zen-4 genetic mutants and RNAi have vulvaless phenotypes, suggesting a novel regulatory function of ZEN-4. We hypothesize that ZEN-4 positively regulates LET-23/EGFR signaling by promoting basolateral localization of LET-23/EGFR. To determine ZEN-4’s relative position in the LET-23/EGFR signaling pathway, we performed genetic epistasis experiments of zen-4 with LET-23/EGFR negative regulators lin-1 and lin-15A/B. It was found that zen-4(RNAi) does not suppress the multivulva phenotypes of both lin-1(n304) and lin-15A/B(n765), suggesting that ZEN-4 functions upstream of the entire LET-23/EGFR pathway. This also indicates that ZEN-4 does not regulate cytokinesis downstream of VPC induction. In order to investigate potential proteins which function with ZEN-4, we then tested whether the other centralspindlin complex subunit CYK-4 [3] also regulates LET-23/EGFR. Observations of cyk-4 mutants and RNAi not exhibiting vulvaless phenotypes suggest CYK-4 to not be involved in the LET-23/EGFR pathway. Overall, these initial results point to a novel centralspindlin-independent function of ZEN-4 promoting LET-23/EGFR signaling upstream of the pathway. Elucidating the mechanism of this regulation will advance fundamental knowledge of EGFR in humans and lead to new approaches of pathway manipulation for disease therapy. References: [1] Sigismund, Avanzato and Lanzetti, Mol Oncol, 2018. [2] Thomas and Weihua, Front Oncol, 2019. [3] Pintard and Bowerman, Genetics, 2019. [4] Portereiko et al., Curr Biol, 2004. [5] Von Stetina et al., Mol. Biol. Cell, 2017.

P1391

Shaping the basement membrane through kinesin-3 and kinesin-1 driven polarized secretion
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The sorting of newly synthesized proteins into apical or basolateral secretory pathways is an important step in maintaining epithelial cell polarity. Although the basolateral secretory pathway handles many different cargo proteins that ultimately populate distinct basal or lateral plasma membrane domains, little is known about the role of targeted secretion at subdomains within the larger basolateral plasma membrane. One developmentally important group of basolateral cargos is the components of the basement membrane (BM). The BM is a dense, sheet-like extracellular matrix that exclusively lines the basal surface of epithelial cells, where it provides an attachment site for cells, a reservoir of growth factors, and mechanical strength that can guide tissue morphogenesis. Since BM proteins are designed to form networks upon exposure to the extracellular environment, control over the site of their secretion may be particularly important. Working in the follicular epithelium of Drosophila, our lab previously identified the GTPase Rab10 as a key regulator of polarized BM secretion that regulates basolateral sorting of BM proteins and their secretion to the lateral plasma membrane. We have now used a combination of genetics and in vivo live imaging to identify the site of BM secretion and determine how this site is selected. Rab10-based BM secretion is concentrated on the basal-most ~1um of the lateral plasma membrane and the basal surface. This bias requires the activity of two kinesins, kinesin-1 and the kinesin-3 Khc73, which transport Rab10+ vesicles along the polarized microtubule
(MT) network towards the basal surface prior to secretion. Comparison of BM secretion defects and Rab10+ vesicle motility in different motor mutant backgrounds shows that both kinesins are needed to fine-tune the site of BM secretion. When this kinesin-based transport is lost, a network of BM proteins forms in between cells, interfering with normal cell organization, cell movements, and ultimately the final shape of the tissue. These findings highlight the importance of controlling the secretion site for BM proteins, and suggest a better understanding of the role of MT motor-driven transport for different cargos in epithelial cells will enhance our understanding of how diverse epithelial tissues establish and maintain their polarized domains during development.

P1392

Pathogenic Mutations in the Chromokinesin KIF22 Disrupt Anaphase Chromosome Segregation

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KIF22 (or Kid) is a kinesin motor that contributes to the movement of mitotic chromosomes. Point mutations in KIF22 dominantly cause spondyloepimetaphyseal dysplasia with joint laxity-leptodactyl type (SEMDJL2), a skeletal developmental disorder characterized by short limb bones and short stature. Published analyses of SEMDJL2 patient samples identified mutations in proline 148 and arginine 149 of the motor domain as causative of disease pathology and predicted that these are loss of function mutations that inactivate KIF22. We report the identification of a new skeletal dysplasia patient with a point mutation in the coiled-coil rather than motor domain of KIF22. To investigate whether mutations that cause SEMDJL2 affect the function of KIF22 in mitosis, we have built stable cell lines that permit inducible expression of KIF22-GFP with pathogenic mutations R149Q (motor domain) and V475G (coiled-coil domain). KIF22 uses plus end-directed motility and direct binding to chromosome arms to contribute to chromosome congression and alignment in metaphase. Surprisingly, KIF22 with R149Q or V475G mutations is capable of generating forces to move chromosomes toward microtubule plus ends in prometaphase, indicating that mutant motors are active and that R149Q and V475G are not simply loss of function mutations. As cells proceed through mitosis, however, we observe a dramatic defect in anaphase chromosome segregation in cells expressing KIF22-GFP R149Q or V475G. Specifically, live imaging demonstrates chromosome recongression: chromosomes begin to segregate in anaphase, then reverse direction to move back toward the center of the spindle rather than continuing toward the spindle poles before decondensing. This phenotype could be explained by a failure of KIF22 to inactivate in anaphase. Continued generation of forces toward the plus ends of microtubules could cause chromosomes to move back toward the center of the spindle rather than segregating to the poles. Consistent with this model, a phosphomimetic mutation within the tail of KIF22 (T463D), which constitutively activates the motor, phenocopied the effects of SEMDJL2 causative mutations. The observed anaphase defects cause reduced proliferation, abnormal daughter cell nuclear morphology and, in a subset of cells, cytokinesis failure. We are currently exploring mechanisms by which KIF22 inactivation may occur, as well as how these defects may affect chondrocyte proliferation and function, specifically, as these cells set the scale of skeletal development.
Perxisomes

P1393

Organelle “Hugging” - Regulation of Peroxisome-ER Interactions by Phosphorylation in Mammalian Cells
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Many organelles collaborate with each other to fulfil their metabolic and cellular functions. For instance, the synthesis of plasmalogens, an essential lipid of myelin sheaths, is initiated in peroxisomes and finished in the endoplasmic reticulum (ER). Therefore, efficient inter-organelle communication and metabolite transfer is essential. Effective exchange between organelles is facilitated at areas where organelles physically interact through tethering complexes, which bridge the opposing organelle membranes. However, these membrane contact sites (MCS) are not static. The various functions of each organelle require the tethers to be dynamic, e.g. to allow organelle movement; therefore, the tethers need to be tightly regulated, though knowledge on the regulation of the majority of MCS is still lacking. We discovered that the peroxisome-ER MCS, mediated by the peroxisomal ACBD4 and ACBD5 [acyl-CoA binding domain proteins 4/5] that interact with ER-resident VAPB [VAMP-associated protein B], is regulated by phosphorylation. We revealed that various phosphorylation sites in ACBD4 and ACBD5 affect their interaction with VAPB by either decreasing or increasing the binding, and thus regulate peroxisome-ER tethering. The VAP protein family tethers the ER to many organelles via their MSP [major sperm protein] domain, which interact with proteins containing a FFAT [two phenylalanines (FF) in an Acidic Tract] (-like) motif, as present in ACBD4/5. We demonstrated that a phosphomimetic substitution in the FFAT-like motif abolishes the binding of both ACBD4 and ACBD5 to VAPB, and reduces peroxisome-ER contacts. Since this residue is conserved in several FFAT motif-containing proteins, our data also provide insights into how VAPB binding to other FFAT motif-containing proteins may be regulated by phosphorylation. Initial data reveal how kinases/phosphatases act on the ACBD5-VAPB tether. We are currently studying how different conditions such as lipid availability modulate the peroxisome-ER interaction (“hugging”). Our results reveal the first molecular mechanism for the regulation of peroxisomal MCS in mammalian cells and also elaborate on the current FFAT motif-VAP interaction model.

P1394

Peroxisomes form intralumenal vesicles to import fatty acids and compartmentalize proteins in Arabidopsis
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Peroxisomes, which house vital and often oxidative metabolic reactions in eukaryotes, are typically described as small spherical organelles with a single delimiting membrane. Although occasional peroxisomal membrane complexity has been observed in yeast, mammals, and plants, technical challenges have limited the recognition and understanding of this complexity. We developed fluorescent reporters to simultaneously label Arabidopsis peroxisomal membranes and lumen, and we examined these lines using live-cell imaging. Exploiting the unusually large size of Arabidopsis seedling peroxisomes, we discovered extensive and pervasive peroxisomal inner membranes. We found that
peroxisomal internal vesicles accumulate over time, use ESCRT (endosomal sorting complexes required for transport) machinery for formation, and are likely derived from the outer peroxisomal membrane. Moreover, these vesicles can harbor distinct proteins and do not form normally when fatty acid β-oxidation, a core function of peroxisomes, is impaired. This unanticipated structural complexity necessitates revisiting the classical view of peroxisomes as simple, single-membrane bound organelles.

P1395

Characterization of a novel peroxin in Trypanosoma brucei
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Peroxisomes are ubiquitous membrane bound organelles with multiple functions including the degradation of hydrogen peroxide and the oxidation of fatty acids. Kinetoplastids have specialized peroxisomes called glycosomes that are essential. Glycosomes are unique in that they compartmentalize the first seven steps of glycolysis and are indispensable. Proteins known as peroxins (Pexs) coordinate peroxisome and glycosome biogenesis. While their function is often conserved across species, their sequences usually are not. Pexs regulate many processes including peroxisome/glycosome formation and proliferation, and the import of membrane and matrix proteins. In kinetoplastids, homologs for only a small number of known Pexs have been characterized, suggesting that there are more Pexs to be discovered. We are interested in identifying and characterizing additional Pexs. Pex19 is a soluble chaperone that delivers peroxisome membrane proteins, many of which are Pexs, to the peroxisome via interactions with the glycosome membrane protein Pex3. We queried the trypanosome genome for open reading frames that contain a Pex19 binding domain that is present yeast, mammal, and plant in Pex3. We found a single ORF (Tb927.9.11350) containing a putative Pex19 binding domain (P19BD; SNKLEIWEDLKIISFTR), which is conserved in all kinetoplastids. In pursuit of resolving the function of this putative peroxin-like protein, PLP, we have defined its cellular localization, purified organelles using epitope-tagged PLP, identified putative PLP binding proteins via co-immunoprecipitation (co-IP), and shown a growth defect and morphology changes using RNAi. In the kinetoplastid parasite, Trypanosoma brucei, immunofluorescence assays revealed that staining for hemagglutinin antigen-tagged TbPLP (HA-TbPLP) overlapped with the glycosome protein aldolase. Western analysis of organelles isolated via immunoaffinity using HA-TbPLP revealed the presence of multiple glycosome proteins and co-IP experiments demonstrated that TbPLP interacts with proteins involved in glycosome protein import. Protein level modulation through RNAi has shown growth defects in induced cell lines when compared to the tagged cells line, as well as severe impacts on cell morphology when the PLP is knocked down. Phenotypic characterization of PLP-deficient cells is ongoing and will provide insight into the role this protein plays in glycosome biogenesis. Inhibition of glycosome protein import is lethal to trypanosomes. The identification of additional Pexs in these parasites provides a foothold into future investigations into the pathways that coordinate glycosome biogenesis, which may be exploited for pragmatic gain.

P1396

Competition between distinct Pex30 complexes at multiple membrane contact sites regulate organelle homeostasis
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Lipid droplets (LD) and peroxisomes are ubiquitous organelles with central roles in cellular metabolism. These organelles form at discrete domains of the endoplasmic reticulum (ER) and once they mature often remain in close proximity of the ER at membrane contact sites (MCS). The ER domains involved in the formation and MCS with LDs and peroxisomes are enriched in the membrane protein Pex30. Consistent with a role in the process, pex30Δ cells show defects in LD and peroxisome biogenesis. However, how the activity and the targeting of Pex30 to ER MCS with different organelles are regulated remains unknown. Here, we show that the function of Pex30 at different MCS is regulated by its related proteins Pex28, Pex29 and Pex32. These proteins assemble with Pex30 in two distinct complexes: one containing Pex30-Pex28-Pex32 that targets ER-peroxisome MCS, and another containing Pex30-Pex29 enriched at the nuclear membrane-vacuole junction (NVJ), a distinct MCS where a population of highly metabolically active LDs concentrates. Thus, Pex28, Pex29 and Pex32 serve as MCS adaptors conferring specificity to Pex30. The absence of any of these adaptors results in defects in the number and spatial distribution of LDs or peroxisomes in the cell. These adaptors compete for binding to Pex30, suggesting that the coordination of LD and peroxisome biogenesis is maintained by the relative abundance of these complexes. We propose Pex30 complexes as general regulators that define organelle biogenesis sites and regulate organelle abundance, and ultimately are responsible for maintaining membrane and lipid homeostasis.

**Plasma Membrane and Signaling Waves in Migration**

P1397

**Arfgap1 regulates collective cell migration in vivo.**

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Collective cell migration plays important roles in morphogenesis and embryonic development and is a main feature of the formation of metastases in several cancers. Unlike single cell migration, collective cell migration is characterized by cell-cell adhesion and cell-cell communication. We have previously demonstrated that vesicular trafficking plays a critical role in cell guidance and cell-cell communication during collective cell migration. A recent screen aimed at identifying new regulators of vesicular trafficking involved in collective migration identified ArfGAP1 as a regulator of border cell migration in the *Drosophila* ovary. Between stages 9 and 10 of the *Drosophila* egg chamber development, the so-called border cells form a cluster that is attracted by the oocyte through the secretion of ligands to receptor tyrosine kinases (RTKs). We found that the depletion of ArfGAP1 specifically in border cells induces migration defects. Clusters devoid of ArfGAP1 are able to initiate their migration, but loose directionality. We are currently investigating the cause of this phenotype by analyzing various determinants of border cell migration. Our initial results indicate that the depletion of ArfGAP1 reduces the level of active RTKs at the plasma membrane, as they accumulate in a late endosomal compartment. Our results suggest that ArfGAP1 is necessary for the proper sorting of active RTKs in endosome and possibly their recycling to the plasma membrane. We identified ArfGAP1 as a new regulator of border cell migration that might acting through vesicular trafficking to maintain of active receptor tyrosine kinases at the plasma membrane. This study could potentially reveal a new important mechanism in collective cell migration, and by extent in cancer dissemination.
Spatial segregation of plasma membrane signaling components in the blebs during leader-bleb based migration

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Cancer cells migrating in a confined tissue microenvironment can switch between different modes of migration depending on the degree of confinement and availability of adhesive ligands. Cells with high contractility are prone to blebbing and when confined in the absence of adhesive ligands, exhibit “leader bleb-based” (LBBM) migration. This mode of migration is characterized by a highly polarized morphology with a long, stable leader bleb (LB) leading the direction of migration, that is separated from the trailing cell body (CB) by a narrow contractile neck. This rapid mode of migration is mediated by fast actin retrograde flow (~9 microns/min) from the bleb tip to the bleb neck. However, the mechanisms mediating the establishment and maintenance of the extreme cell polarization characterizing LBBM are not known. We sought to test the hypothesis that polarization of LBBM is mediated by the segregation of signaling components on the plasma membrane (PM). To test this, we imaged A375M2 metastatic cancer cells with transiently transfected fluorescent probes under 3μm confinement with an agarose pad. We performed time-lapse confocal, super-resolution microscopy, and FRAP to analyze the spatial distribution and dynamics of fluorescently-tagged membrane proteins of specific topology in leader blebs. This showed that proteins on the outer leaflet and the inner leaflet are distributed uniformly throughout the bleb but the intensity goes down around the neck. In contrast, transmembrane proteins exhibit a strong gradient with accumulation at the base of the bleb compared to the bleb tip. FRAP photobleaching experiments showed that proteins with different topological features show restricted diffusion between LB and CB. With live imaging of actin, we show that the tip of the bleb is decorated with bundles of filamentous actin while the back of the bleb has more actin meshwork. Disrupting actin retrograde flow by actin stabilization or reducing myosin II activity changes the segregation of transmembrane proteins. To address the polarity signature of leader bleb based migration, we looked at the distribution of biosensors for PIP2 (PLCδ PH-GFP) and PIP3 (Akt PH GFP). PLCδ PH-GFP labeled the PM evenly and Akt-GFP was excluded from the blebs. Similarly, RhoA GTPase biosensor (rhotenkin-GBD-GFP) labeled tips of the leader blebs while Rac GTPase biosensor (Pak-GBD-GFP) accumulated on the PM of the cell body. All the above data together suggest that there is plasma membrane compartmentalization between LB and CB. The signaling of the PM during LBBM is highly polarized and is different from the front-rear polarity as in adhesion based migration. We are currently testing the hypothesis that cytoskeletal-driven active processes drive segregation of the signaling components differentially on the PM.

Differential Surface Charge on the Plasma Membrane Polarizes Cells During Migration

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In migrating cells, components and activities of the signal transduction excitable network (STEN) such as phosphoinositides, Ras and PI3K self-organize on the plasma membrane and propagate as waves. Such self-organization of network molecules leads to the generation of activated (or ‘front’) and inactivated (or ‘back’) regions on the cell cortex, thereby establishing polarity and guiding cell migration. Although biochemical interactions amongst these network components are often studied, the biophysical principle governing their self-organization on the membrane has remained unexplored. Our studies in Dictyostelium amoeba and RAW 264.7 monocyte/macrophage-like cells showed that negatively-charged phospholipids, including PI(4,5)P2 and phosphatidylserine, organize to the back region of the cell membrane, suggesting that it is more negatively charged than the front. Consistently, a polycationic surface charge sensor organized on the cell membrane in a similar pattern. To assess the role of the surface charge, we designed actuators to abruptly alter it. First, we transiently shielded the negative charge in the back region by optogenetically recruiting a polycationic peptide to the membrane, which resulted in de novo generation of localized protrusions in Dictyostelium and HL-60 neutrophil-like cells. Furthermore, shielding the surface charge by loading the cytosol with calcium ions resulted in increased protrusive activity all over the cell perimeter. Second, we increased the negative surface charge by recruiting polyanionic peptides. In RAW 264.7 cells, exposed to uniform chemoattractant, quietening of protrusive activity at the site of actuator recruitment led to and migration in the opposite direction. In MCF10A epithelial cells uniform membrane recruitment of the polyanionic peptide caused inhibition of Ras and ERK. Our study has uncovered a biophysical mechanism by which STEN molecules are arranged within phase domains on the plasma membrane of a migrating cell by virtue of the subtle difference in negative surface charge on the inner leaflet.

P1400

Diffusion mediated phase separation enables symmetry breaking of membrane protein distributions during excitable cortical wave propagation and macropinocytosis

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Excitable waves of coupled signaling and cytoskeletal activities are emerging as a crucial underlying organizer of macropinocytosis, phagocytosis, cytokinesis, and amoeboid migration. Typically, wave propagation reflects the sequential, reversible membrane-cytosol shuttling of peripheral membrane proteins or biosensors, reminiscent of a “stadium wave”. Hence, constitutively bound non-shuttling membrane proteins are generally considered to be symmetrically distributed and are not expected to participate in similar stadium waves. However, we have observed that a few different lipidated membrane proteins such as Gβγ, PKBR1, and different Ras (and Rac) proteins exhibit distinct symmetry breaking, dynamically localize in specific regions of the cortex, and propagate as waves. This novel symmetry breaking implied that amoeboid cells possibly tightly confine most of the inactivated form of the “front” signaling proteins to the so-called “back” region of the cortex to spatiotemporally limit the “front”/protrusion area. Further investigation on this unexpected pattern formation revealed a new mode of excitable wave propagation: instead of shutting, this wave effect is brought about by lateral
translocation and dynamic partitioning. Diffusion gradient and/or differential reaction with separated phase states of cell cortex can create definite compartmentalization of these proteins. Finally, using Unstructured Reaction-Diffusion Master Equation (URDME) formalism based two-dimensional simulation of excitable network, we have established that either shuttling or partitioning can yield waves with extremely similar length and time scale, as we have observed experimentally.

Recycling Pathway

P1401

**Loss of CLC-5 alters endocytic traffic and cholesterol distribution in the kidney proximal tubule**

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Dent disease is a progressive X-linked disorder caused by loss of function of the Cl−/H+ exchanger, CLC-5 which is expressed at the plasma membrane and early endosomes in epithelial cells of the kidney proximal tubule (PT). Early symptoms of Dent disease include low molecular weight (LMW) proteinuria resulting from inefficient apical endocytic recovery of filtered proteins by PT megalin and cubilin receptors. Knockout of Clc-5 in mice recapitulates the LMW proteinuria observed in human disease and causes reduced expression of megalin and cubulin protein but not mRNA. Previous microarray studies in Clcn5 KO mice suggest alterations in the expression of cholesterol and lipid metabolic pathways. We hypothesized that changes in lipid homeostasis or dynamics contribute to reduced receptor protein expression in Dent disease. We investigated the role of Clc-5 in megalin traffic in siRNA knockdown (KD) and CRISPR/Cas9 Clc-5 knockout (KO) polarized PT cells, and in CRISPR/Cas9-meditated Clc-5 KO mice. Megalin had a shorter half-life in Clc-5 KO cells compared with control, and there was a reduced fraction of total megalin at the apical surface, consistent with reduced efficiency of receptor recycling. Additionally, we observed an apparent accumulation and redistribution of cholesterol in both Clc-5 KD cells and in the PT of heterozygous Clc-5 KO mice compared with controls. Current studies are focused on quantifying the recycling kinetics and distribution of megalin in PT cells in the absence of Clc-5 and determining whether and how altered cholesterol metabolism contributes to the reduced endocytic capacity of the PT and resulting LMW proteinuria in Dent disease.

P1402

**Ubiquitination of Ist1 controls surface protein recycling from endosomes**

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Cell surface membrane protein cargoes coordinate a myriad of essential cellular functions, from transport to signalling these surface cargoes are regulated via dynamic membrane trafficking events in the endomembrane system. Although internalised cargoes are known to recycle back to the surface via various distinct pathways, the regulatory mechanisms of these pathways, the organisation of the endomembrane system, and the division of labour between them is not fully understood. In yeast, these trafficking events are even less clear, but we establish two classes of cargo that rely on distinct recycling mechanisms. Using time-lapse 3D confocal microscopy coupled to microfluidics and photoconvertible labelled surface cargoes, we find that substrate induced endocytosis of nutrient transporters occurs
rapidly to a Vps4/Ist1 compartment. We show recycling can occur from these endosomal compartments, which we believe are distinct from multivesicular bodies. A genome-wide screen for recycling machinery identified Ist1 as required for recycling, which is confirmed using multiple assays. We propose that Ist1 polymerisation is required for scission of recycling intermediates and show Ist1 is ubiquitinated, which is required for recycling. We propose Ist1 ubiquitination allows recruitment of the AAA+-ATPase Cdc48 to recycling endosomes via the adaptor Npl4, which was also identified in the genetic screen for recycling machinery, to drive depolymerisation and allow further scission events. Collectively these results show that surface recycling directly from endosomes in yeast relies on the precise regulation of Ist1, which conceptually aligns with other in vitro and in vivo models.

**P1403**

*Positive regulation of the Retriever recycling pathway by the lipid kinases Vps34 and PIKfyve*

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SNX17, Retriever, the actin‐regulatory WASH, and COMMD/CCDC22/CCDC93 (CCC) complexes, act together to recycle proteins from endosomes back to the plasma membrane. This recycling pathway transports multiple receptors including those that are critical for cell migration and cholesterol homeostasis. Little is known about how this pathway is controlled. Here we discover that the phosphoinositide lipid kinases, Vps34 and PIKfyve positively regulate this pathway. Vps34 is a lipid kinase responsible for the generation of the signaling lipid phosphatidylinositol 3‐phosphate (PI3P) at endosomes. We find that inhibition of Vps34 inhibits the association of SNX17, the WASH, CCC and Retriever complexes to endosomes. SNX17 and the WASH subunit, FAM21 directly bind PI3P, which suggests that Vps34 initiates this pathway by recruiting these proteins. SNX17 also binds directly to its cargoes. The PI3P generated by Vps34 is also responsible for a pool of the signaling lipid phosphatidylinositol 3,5-bisphosphate (PI3,5P2) via PIKfyve, a PI3P 5-kinase. PI3,5P2 is also converted by myotubularin-related phosphatases to most of the cellular phosphatidylinositol-5-phosphate (PI5P) pools. The best characterized roles of PIKfyve are in lysosome homeostasis. Here we show that PIKfyve colocalizes with SNX17, Retriever, WASH and the CCC complex on endosomes. Further, we show that PIKfyve controls recycling of cargoes of the SNX17‐Retriever‐CCC‐WASH pathway including integrins, key players in cell migration. Importantly, inhibition of PIKfyve causes a loss of both CCC and Retriever subunits, and a concomitant accumulation of SNX17 on endosomes. PI3,5P2 and/or PI5P likely directly recruit the CCC complex because some CCC subunits bind these lipids in vitro. The Retriever complex may also be recruited via these lipids, and/or may be recruited via the CCC complex. Together, these studies suggest that PI3P generated by Vps34 recruits SNX17 bound to its cargo, as well as the actin-regulatory WASH complex. PI3P also recruits PIKfyve via the FYVE domain and this leads to synthesis of PI3,5P2 and PI5P on endosomes. PI3,5P2 and/or PI5P further recruit the CCC complex, which in turn recruits the myotubularin-related phosphatase, MTMR2. The CCC complex along with the WASH complex likely recruit the Retriever complex. Furthermore, a Retriever subunit binds directly to SNX17. This network of connections completes the assembly of the SNX17-WASH-CCC-Retriever complex, and allows receptor recycling to the plasma membrane. Importantly, these studies suggest an ordered pathway that is mediated by progressive generation of phosphoinositide signaling lipids from PI3P to PI3,5P2/PI5P, to regulate the assembly of the SNX17-WASH-CCC-Retriever recycling pathway.
Annular gap junction processing: recycling versus degradation

Gap junction channels play a pivotal role in the communication of molecules between contacting cells. These gap junction channels are composed of proteins called connexins. The assembly and aggregation of channels into to gap junction plaques, and the gap junction plaque internalization process that results in the release of annular gap junctions into the cytoplasm of one of two contacting cells has been well documented. The fate of these annular gap junctions however is poorly understood. The central dogma is that annular gap junction formation is only a mechanism for connexin protein degradation. However, it is becoming clear that annular gap junctions undergo processing events that includes fusions with other organelles, including with the plasma membrane (recycling). To elucidate the fate of annular gap junctions in cells expressing connexin 43 (Cx43), immunocytochemical colocalization techniques and 3D computer-assisted reconstruction were used to analyze the association of annular gap junctions with lysosomal (LAMP), recycling (RAB 11) and retrograde/recycling transport (VPS35) markers. Annular gap junctions were demonstrated to associate with lysosomes, as expected, given the known role of lysosomes in annular gap junction degradation. However, the percentage of annular gap junction colocalization with the degradation marker, LAMP (10.9% ± 2.4%) was less than the percent colocalization with the recycling, RAB 11 (20.9% ± 1.87%) or sorting protein, VPS35 (17.1% ± 1.4%) markers. The finding that annular gap junctions localized with the degradation marker, LAMP, less than with either VPS35 or RAB 11 is consistent with a higher rate of recycling than degradation. It is concluded that while some annular gap junctions, once internalized, fuse with and are degraded by lysosomes, other annular gap junctions recycle their Cx43 proteins back to the plasma membrane potentially for reuse in gap junction channel formation. Supported by NSF grant MCB #2011577

Retromer- and Snx4- independent endosomal recycling in yeast
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Recycling of sorting components (e.g., receptors, etc.) in the endo-lysosome system is essential for normal assembly and function of the lysosome. We have been studying the role of the retromer and the Snx4 complexes for cargo sorting into recycling tubules. During this process, these complexes are recruited to the endosomal surface where they bind cargo containing a specific sorting motif. They deform the membrane into recycling tubules that bud from the endosome and recycle the cargoes to the Golgi or the plasma membrane. Recently, we identified a novel protein coat complex responsible for membrane protein recycling in a retromer- and Snx4- independent manner. Genetic, cell biological, and biochemical analyses revealed how this complex deforms the endosomal membrane and sorts a specific cargo into recycling tubules. I will describe this process and provide a model for why yeast cells require these three distinct recycling complexes.
RNA Regulation of Gametogenesis

P1406

EXOSC10 sculpts the transcriptome during the growth-to-maturation transition in mouse oocytes

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Mammalian oocyte development depends on both female germ-cell growth and maturation. The growth-to-maturation transition begins with germinal vesicle breakdown (GVBD) and is critical for maintaining oocyte quality and competence for early embryonic development. Substantial amounts of RNA accumulate during oocyte growth and most is degraded during subsequent meiotic maturation. Failed degradation of the transcriptome directly results in poor oocyte quality. However, the molecular machinery responsible for degradation of oocyte RNA remains incompletely understood. Here, we report that an exosome-associated RNase, EXOSC10, sculpts the transcriptome to facilitate the growth-to-maturation transition of mouse oocytes. We establish an oocyte-specific conditional knockout of Exosc10 in mice using CRISPR/Cas9, perform multiple single oocyte RNA-seq analyses to document a dysregulated transcriptome, and link changes in the transcriptome to abnormal phenotypes observed in mutant oocytes. We determine that Exosc10 null oocytes exhibit delayed GVBD which results in female mice subfertility. Single oocyte RNA-seq data document dysregulation of multiple types of RNA including mRNAs that encode proteins important for endomembrane trafficking, meiotic cell cycle progression and RNA processing. Thus, EXOSC10-depleted oocytes have impaired endosome maturation, decreased endoplasmic reticulum levels, increased quantities of lysosomes and abnormal distribution of their Golgi. In addition, CDK1 fails to activate, possibly due to persistent WEE1 activity, which blocks lamina phosphorylation and nuclear envelope disassembly. Moreover, we identify rRNA processing defects that result in a higher percentage of developmentally incompetent oocytes after EXOSC10 depletion. Collectively, we conclude that EXOSC10 promotes normal growth-to-maturation transition in mouse oocytes by processing rRNAs and degrading RNAs encoding growth-phase factors to support the maturation of oocytes. This study provides insight into molecular mechanisms underlying the maintenance of mammalian oocyte quality.

P1407

Examining the interplay between sRNA pathways and germ granules in Caenorhabditis elegans

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Small RNA (sRNA) pathways are critical regulators of gene expression, germ cell integrity, and fertility. These pathways involve the coordination between sRNAs and their binding partners, the Argonaute (AGO) proteins. C. elegans contains 21 different AGOs. Our lab has systematically examined all 21 AGO expression patterns and observed that eight AGOs localize to germ granules. Germ granules are phase separated RNA and protein rich organelles that are only present in germline cells, and are conserved across species. Four different germ granules (P granules, Z granules, Mutator foci, and SIMR foci) have been observed in C. elegans germline and all four appear to perform separate functions. Germ granules can either be distributed in the cytoplasm, or they can associate with the nuclear periphery and nuclear pores. This association means that over 75% of transcripts in the germline pass through germ granules. All three types of germ granules contain proteins that are involved in RNA metabolism and sRNA
pathways. Disruption of the germ granules leads to phenotypes that implicate germ granules in numerous processes including RNA processing, transgenerational RNA interference (RNAi) and fertility. We aim to identify which AGOs and germline transcripts localize to each of the granules, and how AGOs and the different germ granules interact to carry out their functions. For example, CSR-1 is an essential AGO which is known to localize to P granules thus we examined the localization of one of its known target transcripts, pgl-3, using smFISH to identify its localization. We saw that the pgl-3 transcript localized to germ granules with partial overlap with CSR-1, and that this localization was disrupted in sRNA pathway, germ granule and nuclear pore mutants. While the few AGOs that were previously shown to localize to germ granules are mainly associated with one type of granule, we are now in the midst of examining these patterns more carefully using quantitative co-localization with AGO-tagged and germ granule-tagged strains. Overall our studies will delineate the functions of each type of germ granule and will uncover new links between small RNA biology and germ granules that are important for epigenetic inheritance and fertility.

P1408

**Structural and molecular determinants of dynamics in P granules of *C. elegans***

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The germline of sexually reproducing animals contains conserved non-membrane-bound compartments containing proteins and RNAs called ‘germ granules’. Mutations that disrupt assembly of germ granules result in sterility. A germ granule in *C. elegans*, called ‘P granules’, has been shown to assemble via liquid-liquid phase separation (LLPS) of proteins and RNAs from the surrounding cytoplasm in adult gonads. Approximately 85% of proteins that concentrate in P granules are involved in different aspects of RNA metabolism, suggesting that the P granule phase could process and/or store RNA. To elucidate the underlying molecular mechanisms, it is important to understand the biophysical nature of the P granule phase. Specifically, the molecular and structural determinants that regulate diffusion rates within the P granule phase remains unclear. We used an in vitro reconstitution-based approach to address this question and studied the LLPS behavior of the protein PGL-3, one of the most abundant proteins in P granules. We found that a folded domain of PGL-3 drives LLPS, and dynamics within phase-separated condensates correlates with alpha-helicity of the folded domain. Next, we investigated the effect on dynamics in condensates when other components of P granules are added to condensates of PGL-3. Our findings suggest that the complex composition of P granules favor fast dynamics within the phase.

P1409

**Gla-3/tristetraprolin plays an important role in the germline stress response of *caenorhabditis elegans***

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GLA-3 is the *C. elegans* homolog of the mammalian tristetraproline (TTP), an RNA binding protein with two CCCH-like zinc-finger domains. In mammals, this protein interacts with ARE-containing elements present in mRNAs to promote their degradation, and during stressful conditions associates to stress granules (SG). SGs are dynamic cytoplasmic membrane-less organelles that are formed by liquid-liquid phase separation. These organelles are composed of mRNAs that are stalled in translation pre-initiation complexes and different types of mRNA binding proteins like TTP and TIA-1. In mammals TTP is phosphorylated by ERK to promote SG formation. In *C. elegans*, GLA-3 is expressed in both the soma and germline. *gla-3* mutant animals show different phenotypes among them are: protein degradation in muscle leading to a progressive loss of motility, severe defects in meiotic progression, increased germ cell apoptosis, reduced brood size and embryonic lethality in low frequency. It was shown, by immunoprecipitation, that GLA-3/TTP associates with the MAP kinase protein MPK-1/ERK, which in the worm is required for pachytene progression during oogenesis. The role of the interaction between GLA-3/TTP and MPK-1/ERK in this organism it not known yet. Our aim is to study the function of GLA-3 in the germline in normal and stressful conditions. The adult hermaphrodite *C. elegans* gonad is a syncytium in which sperm and oocytes are produced. It has been estimated that during oogenesis fifty percent of germ cells served as nurse cells to later being eliminated by apoptosis. We observed that when we exposed young adult hermaphrodites to 6 h of starvation (bacterial deprivation) or 3h of heat shock (31º C) germ cell apoptosis increases, and ribonucleoprotein (RNP) complexes, similar to SG, are formed in the gonad. We have identified two main classes of SG in the gonad: one that is formed in the core and another in the proximal oocytes. We found that GLA-3/TTP is required to induce germ cell apoptosis during starvation and heat shock. Using the transgene *gla-3a (tn1734[gfp::3xflag::gla-3a])* (made by the Greestein lab), we observed that the fusion protein GLA-3::GFP associates to SG during heat shock. Furthermore, we observed that *gla-3* mutant animals are unable to form SG in their gonads during heat shock, oxidative stress or starvation suggesting that this protein is important for the association of these RNP complexes. Silencing of *mkp-1/ERK* prevented the formation of SG suggesting that this protein might be important to regulate SG formation through GLA-3/TTP. We concluded that GLA-3/TTP plays an important role in germ cell protection against stress. We are grateful to PAPIIT/DGAPA (grant number: IN208918) for financial support.

**Science Education: Innovations in Teaching**

P1410

**Assembly of protein complexes using 3D models printed in soft silicone**

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**Background.** All life forms are supported by the work of proteins, very large macromolecules that exert their function through interactions and dynamic activity. Protein structures are determined through a growing number of techniques (based on X-ray, Cryo-EM and NMR), providing data on the general shape of proteins (and sometimes on their movements), deposited in the dataBank as atomic coordinates. Reading 3D coordinates is accomplished through one of few software that transform them into virtual images, using techniques of 3D computer graphics. Some authors have proposed the fabrication of protein models using direct printing or other techniques, but a general method for producing models...
that can be adopted in most settings was still lacking. **Results.** We propose a general pipeline for casting flexible protein models in soft silicone, based on the PDB files and on the design and printing of a two-pieces mold. The bipartite mold is automatically generated by an algorithm taking into account the geometrical and topological properties of the protein, so that the latter can easily be extracted once it is cast. We have evaluated our pipeline on We present our results multimeric Actin, tetrameric Hemoglobin and the Histone Octamer. All models can be fabricated as monomers (dimers in the case of Histones) and their assembly can be facilitated by the insertion of magnets at the protein-protein interfaces. **Conclusions.** The availability of physical models of proteins produced as soft, flexible objects makes it possible to handle the monomers and combine them to show their biological assemblies. The procedure for designing the mold, the printing, and the casting details are described; the STL files for the three selected proteins are freely available and deposited on the NIH database. The use of soft protein models will allow a better understanding of the way proteins work, facilitate teaching at high and superior grade courses and possibly inspire biologically based reasoning in any interested people, both scholars and lay persons.

P1411

**Engaging Undergraduate Student Leaders in Developing Scientist Spotlights that Shift Biology Students’ Scientist Relatability and Perceptions of Scientists**

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While scientists struggle to promote inclusion, student leaders are underappreciated partners in undergraduate science education reform, bringing cultural assets and perspectives largely unrepresented among science faculty. As such, we have engaged biology student leaders of color in curricular transformations in the context of a biology service-learning course, to integrate their efforts into their academic degrees. Specifically, we developed the 4-unit upper division biology elective - LEADS: Learners Engaged in Advocating for Diversity in Science. In its initial offering, the LEADS course partnered 11 biology faculty teaching 8 courses with 16 upper-division biology majors of color. Together these LEADS teams developed Scientist Spotlights - brief curricular supplements implemented as homework assignments that highlighted scientists from diverse backgrounds and addressed biology content - for each course. As part of these assignments, enrolled students metacognitively reflected on the stories of these scientists and what that suggested to them about the types of people that do science. To examine the impact of these Scientist Spotlight interventions on students enrolled in the eight courses and those in the LEADS course, we analyzed pre- and post-assessment responses about: their ideas on the types of people who do science, and whether they knew an important scientist to whom they could relate. Four major findings emerged: 1) Students (n=752) that completed as few as 3 Scientist Spotlights had significant shifts in their perceptions of scientists, in particular decreased stereotypes about scientists (p<0.001). 2) Scientist Spotlight interventions shifted students’ relatability to scientists in both white students (p<0.001) and students of color (p<0.001). 3) Authoring Scientist Spotlights by LEADS students also resulted in a dramatic 90% increase in their relatability to scientists. 4) There were statistically significant differences on an independent department-wide survey between
students who experienced biology courses with Scientist Spotlights and those who did not about whether they had studied multiple scientists (p<0.001), women scientists (p<0.001), and scientists of color (p<0.001). Taken together, these results suggest that this novel LEADS biology service-learning course positively influenced perceptions of and relatability to scientists among these student leaders and hundreds of enrolled biology students across the department. Engaging student leaders not only as authors of inclusive curricular materials, but also as partners in institutional reform is a novel approach to promoting inclusion and addressing unfair representation in science courses. This effort was supported by an HHMI Inclusive Excellence Award.

P1412

Analysis of Internalized Bias Among Underrepresented Students of Biology

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Science, in service of the society’s greater good, gleans an improved quality of scientific insight when multiple perspectives are freely shared. Paradoxically, representation within STEM disciplines, in its current state, does not fully reflect the human diversity of the society it aims to serve. Among these underrepresented people groups in science and technology are Christians, who depart the STEM educational pipeline at various stages. Previous studies have shown that undergraduate students perceive a robust anti-Christian bias, and direct experiments revealed that an anti-Christian bias indeed exists against evangelical students. Since stereotype threat can greatly influence the performance and persistence of students in STEM disciplines, this current research project unpacks whether anti-Christian biases are internalized by undergraduate evangelical Christian students majoring in biology. We probed, do Biology majors at a Christian college hold pro- or anti-Christian biases in science? How does this potential bias differ developmentally among first year students vs upper-level students? We recreated a previously published audit study in which students enrolled in 100- (N = 70) and 300-level (N = 51) biology courses at a private Christian liberal arts college were asked to evaluate the profiles of fictitious applicants to a Ph.D. program. The mock profiles were identical, save for a disclosure of religious identity (Profile 1 = president of Student Christian Association; Profile 2 = president of Student Activities Association). The surveys were administered in the context of coursework within the undergraduate biology curriculum, and included measures for competence, hireability, and likeability of the mock profiles. Results show that the 100-level distribution of student ratings was nearly identical for all measures, indicating no statistically significant difference in average composite scores for competence, hireability, and likeability between Profile 1 and Profile 2 (Student’s T p>0.01). However, this lack of perceived bias seems to adjust developmentally over the course of student education. Although 300-level students likewise showed no statistically significant differences in competence and hireability ratings between Profile 1 and 2 (Student's T p>0.01), they rated Profile 1 more favorably for the likeability measure (μ = 5.76 +/- 0.14 SEM Profile 1 vs. 5.20 +/- 0.18 SEM Profile 2; t(50) = 2.48 p = .008 Student’s T; Cohen’s d = 0.69). Taken together, these results suggest that 300-level students in this study population develop a slightly favorable likeability bias toward Christians in STEM that does not exist at earlier educational stages. We discuss implications for the role of undergraduate education in diversifying the STEM workforce.
P1413

Attitudes Towards Student Writing From Introductory Biology Faculty

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Objective: This study aims to demonstrate the complex attitudes of university Biology faculty towards student writing in Introductory Biology courses. Methods: ADAPT is a nationwide, longitudinal, multi-phase study on the pedagogical decision-making process of full-time faculty members in Biology classrooms. In Phase 1 of ADAPT, qualitative narratives were obtained from a nationally representative sample of 40 full-time Introductory Biology professors from eight four-year large universities across the United States via 60-to-90-minute semi-structured interviews. During the interview, a student writing assignment intervention was presented to faculty participants to gauge their insights regarding the feasibility of adopting such a tool in their own classrooms. Results: While 100% of participants addressed the logistics of implementing such an intervention, more than 50% of faculty provided extended perspectives on their perceptions on students writing in Introductory Biology courses. Aside from the daunting task of grading hundreds of writing assignments, faculty also expressed low expectations of STEM student writing ability and interest as well as the availability of other kinds of student data (e.g., exams) that already assess student learning. However, there was still some acknowledgement of the value of developing writing skills for scientific communication purposes and initiatives towards promoting writing development in STEM classrooms. Conclusion: This study concludes that, aside from the challenges of grading large volumes of textual data, faculty perceive that Introductory Biology students lack the skill and ability to write about science effectively. Perhaps promoting a disciplinary culture of valuing scientific writing as part of the curriculum and providing institutional resources for doing so may enhance faculty attitudes towards student writing in Introductory Biology classrooms.

P1414

Perceptions of evolution among Muslim undergraduate biology students in the United States

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Evolution is a core concept of biology, yet many college biology students struggle to accept evolution due to perceived conflicts with their religious beliefs. Previous biology education studies have demonstrated a need for Religious Cultural Competence in Evolution Education (ReCCEE) to increase religious students’ evolution acceptance by decreasing the perceived conflict between evolution and religion. However, within the United States, most studies have only evaluated Christian student challenges to learning and accepting evolution due to conflict with religious beliefs. To our knowledge, no studies to in the United States have examined Muslim student perceptions of conflict between religion and evolution, acceptance of evolution, or understanding of evolution. In this study, we used previously validated survey instruments to examine Muslim students’ evolution acceptance, understanding of evolution, interest in evolution, importance of religion, and perceived conflict between religious beliefs and evolution in introductory biology courses across the United States. We analyzed surveys from 5,059 students in over 50 college biology courses as a part of a larger study examining Religious Cultural Competence in Evolution Education (ReCCEE). Surveys were administered before any
evolution instruction. Analyses indicate that Muslim students are just as religious as non-Catholic Christian students, and that they have similar levels of evolution acceptance, understanding, and perceived conflict between their religious beliefs and evolution as non-Catholic Christian students. For instance, only 36% of Muslim students (n=139) accepted that life on Earth shares a common ancestor similar to that of non-Catholic Christian students. Despite this, Muslim student interest in evolution was higher than that of their non-Catholic Christian peers. This study importantly highlights that Muslim undergraduate biology students are a population that may benefit from religious culturally competent evolution instruction. However, current ReCCEE instruction is built to be culturally competent for Christian students and not Muslim students. Future research is needed to explore how cultural competence in evolution education could be adapted for Muslim student populations.

P1415

Using metacognitive strategies to improve student learning outcomes in an advanced-level biochemistry course
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Evidence suggests that students’ self-beliefs about their academic abilities can directly influence long-term achievement. These self-beliefs, or mindsets, can be described as fixed or growth oriented. Little is known about how these attributes affect student learning at the college level. Here, we utilize metacognitive strategies ranging from growth mindset (GM) messaging and concept mapping to written reflections, exam wrappers, and instructor GM feedback. We hypothesize that metacognitive interventions not only improve GM, but also increase achievement of student learning outcomes. We used a mixed-methods study design over four separate iterations of a one-semester Biochemistry survey course. Students had the same material and assessments. GM was measured using an 8-item pre/post growth mindset inventory (GMI; Dweck). Two sections (n=87) were exposed to GM metacognitive interventions, while the other two sections (n=74) were not (non-GM). Assessment data from in-class exams showed learning gains for all students regardless of intervention. Moreover, the GM of students did not differ (GMI 3.1 + 0.5 SD (GM) vs 3.3 + 1.3 SD (non-GM). Interestingly, there was stronger performance on the cumulative American Chemical Society Biochemistry Exam for students receiving GM interventions (Mean percentile ranking of 52.3% + 3.2 SD (GM) vs 46.2% + 4.2 SD (non-GM); p<0.01). Our findings suggest that metacognitive interventions have a positive effect on student learning.

P1416

Annotation of genetics elements through analysis of multiple contigs in Drosophila ananassae
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Drosophila melanogaster is a well-studied model organism in biology and is used as a reference species for a number of developmental, molecular and cellular processes common to higher eukaryotes. The genome of Drosophila melanogaster was completed in 2000 and it has since been used as a reference for identifying genes and other genomic elements in other Drosophila species. The goal of this project is to analyze three different genomic regions from Drosophila ananassae to identify and annotate protein
coding regions. These regions will include, contig 3 present on the Muller D element, a genomic region which is ~31,000 bp in size, and contigs 48 and 59 on the Muller F element which are ~600,000 bp and ~440,000 bp, respectively. In addition to identification and annotation of the genes within these regions, the goal of this project is to also compare, contrast and highlight unique characteristics present in the protein coding regions, such as nonconsensus intron donors or acceptors, inconsistencies in number of exons, presence of pseudogenes, etc. (if any). Sequence analysis and data collection will be carried out using a number of open-source computational genomic tools for sequence alignment, gene-prediction and *Drosophila* genome browsing. The data files and resources for this project were available through the Genomics Education Partnership (GEP).

P1417

**Niblse collaborative community model for developing, disseminating, and assessing bioinformatics learning resources**

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The Network for Integrating Bioinformatics into Life Sciences Education (NIBLSE) is an NSF-funded Research Coordination Network that promotes bioinformatics as an essential component of undergraduate life sciences education. In a nationwide survey of over 1,200 life science instructors, NIBLSE previously reported that the most commonly cited barrier to the integration of bioinformatics into undergraduate biology classes was faculty training. In response, NIBLSE has cultivated a collaborative community model to facilitate the development, dissemination, and assessment of bioinformatics learning resources. To begin the process, a course instructor(s) who has authored a learning resource addressing one or more NIBLSE bioinformatics core competencies submits their original materials to NIBLSE for wider dissemination in the NIBLSE Learning Resource Collection on QUBES. If requested, a small community of faculty then work collaboratively in a NIBLSE Incubator to refine the resource, typically expanding its suitability for a broader range of classes. Often the revised version of the learning resource goes on to be published in *CourseSource* or a similar peer-reviewed journal. Furthermore, a NIBLSE-incubated resource can be further disseminated in a QUBES Faculty Mentoring Network (FMN), which supports faculty members implementing a resource in classrooms across the nation. Another FMN outcome is that participants may produce adaptations, which can then be linked to the original resource in the NIBLSE collection on QUBES. In summary, the NIBLSE collaborative community model effectively harnesses the intellectual capital of community members to facilitate the development, dissemination, and assessment of learning resources to address common barriers to the integration of bioinformatics into undergraduate life sciences education.

P1418

**Nematode hunters: a citizen-science approach to identifying new systems for the study of host-virus interactions**

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The nematode *Caenorhabditis elegans* is a popular model organism that has proved very useful for studying the cell biology of intracellular infections due to its’ short life cycle, optical transparency, and ease of genetic manipulation. However, the use of *C. elegans* as a model for the study of host-virus interactions has been limited by the fact that only one natural viral pathogen of *C. elegans* has been identified to date (Félix and Wang, 2019; Franz et al., 2014). The goal of this project is to identify novel natural nematode viruses capable of infecting *C. elegans* by mobilizing ordinary citizens to collect wild nematodes. Studying the interactions of different types of viruses with their host’s cells can provide new insights into cell biology and host-pathogen interactions. To date, only four viruses naturally infecting *Caenorhabditis* nematodes have been identified, and of those only one (Orsay virus) infects *C. elegans* (Félix et al., 2011; Frézal et al., 2019). In the past, identification of intracellular pathogens in wild-caught nematodes has relied on detection by microscopy of morphological changes caused by the infection (Félix et al., 2011; Troemel et al., 2008). This approach is relatively low throughput and requires an expert screener. Our approach instead uses a fluorescent reporter-based method, taking advantage of a set of genes which are expressed at low levels in basal conditions but highly upregulated during infection by intracellular pathogens (Bakowski et al., 2014; Reddy et al., 2017). Co-culturing infected nematodes together with *C. elegans* expressing these intracellular infection reporters produces fluorescence which is easily detected on a fluorescence dissecting microscope. By using this method on a large sampling of wild-caught nematodes, we hope to identify novel nematode viruses which can be transmitted to *C. elegans*. In the pilot phase of this project, we established protocols for wild nematode collection which require minimal supplies and can be performed at home by people with no particular science background after viewing a short training video. We have successfully cultured wild nematodes from these samples in the lab, and have established systems for sample intake, expansion and frozen stocking of the strains, performing co-culture experiments, and sharing experimental results with the original collectors. In the future, we hope to expand this project by partnering with educators at a variety of levels who would be interested in incorporating nematode hunting into their science curriculum.

P1419

**Effective mechanisms for engaging ECRs in Higher Education policy training**  
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COVID-19 has caused a lot of misinformation with regards to the value & importance of science in dealing with the pandemic. Additionally, the pandemic-induced lockdown has adversely affected higher education. However, the post-pandemic recovery process is going to be a great opportunity to create reforms in higher education and increase engagement of policymakers and lay audiences on science-related issues. In order to bridge the gap between researchers and policymakers, the JSPG in collaboration with UCI, offered the virtual Science Policy & Advocacy for STEM Scientists Certificate Program. The program equips ECRs with fundamental skills in science policy and advocacy, including effective communication for engaging policymakers on a multitude of issues. Certificate Program learning sessions described effective communication mechanisms and methods for ECRs to engage policymakers. The program also provided ECRs with opportunities for networking, as well as peer-to-peer review and feedback from policy professionals. We plan to organize a similar workshop on science
policy and advocacy training at the ASCB conference, discussing issues related to research funding, higher education & workforce development, and tools for effective advocacy on these topics. Workshop attendees will build policy elevator pitches on higher education related issues & receive feedback in real-time.

P1420

Introduction of Collaborative Learning Sessions on Molecular and Cellular Basis of Disease Significantly Improved Outcomes in a Graduate-Level Physiology Course.
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Physiology is a requisite course for many professional health programs. The spread of COVID-19 presented a complex challenge for many medical educators: rapidly adapt to teach traditionally in-person classes in a virtual format. The purpose of this study was to compare the effectiveness of physiology course offered last year to entry level students in health profession programs in the traditional, in person instructor-led learning format to the same course this year, which was delivered fully online. Both years the course was divided into 4 units, each unit concluded with an exam. In addition, students took the same test as both a pretest in the beginning of the course and as a final test. The comparison was done in terms of knowledge of physiology measured in exam scores and pretest-posttest improvement. Significance of differences between average exam scores was determined with Student's t test. Upon unexpected transition to online mode, the first two course units were delivered as a combination of live synchronous lectures and posted lecture recordings. There was a slight decrease in unit 1 and unit 2 exam scores in some cohorts in comparison to in-person content delivery. In order to prevent further reduction in performance and improve class engagement, we have introduced short (10-15 minutes) collaborative learning sessions in small groups at various times during live online lectures. Typically, a very short time is dedicated to discussion of cell biology in traditional physiology course. However, knowledge of fundamental molecular and cell biology is necessary for understanding of many clinically relevant concepts in modern physiology. Therefore, we decided to focus group sessions on discussion of the molecular and cellular basis of disease, exploring therapeutic targets and formulating hypotheses about potential treatments. Analysis of unit 3 and unit 4 exams revealed that incorporation of small group collaborative learning sessions resulted in significantly higher scores in comparison to traditional in-person didactic lectures (p-value <0.05). Furthermore, this year we observed significantly greater pretest-posttest change (p-value < 0.01), indicating overall improvement in learning outcomes. Altogether, we believe that successful switch to online delivery mode requires some restructuring of the traditional physiology curriculum. In our case incorporation of interactive group learning experience significantly improved students' performance levels. Furthermore, further evaluation of benefit of expansion of clinically relevant cell and molecular biology content can be useful to improve effectiveness of physiology courses offered to health professions students.
Signaling from the PM to the Cytoplasm to the Nucleus

P1421

**Novel Synthetic Small Molecules with Potent Proliferative Activity Stimulate BMP-dependent Signaling Pathways**

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Bone Morphogenetic Proteins (BMPs) play critically important roles throughout embryonic development and in adults regulating diverse cellular processes including proliferation, differentiation and tissue repair. Recent attempts to use recombinant BMPs as therapeutic agents have been compromised with translational limitations such as high production cost, method of delivery and adverse effects. Our lab has recently demonstrated that select members of a novel series of small synthetic compounds sharing a common core structure mimic the actions of BMPs. Two of these series, LB35 and LB57, showed potent proliferative activity in the pluripotent C2C12 cell line. Here, we explored the mechanisms underlying this proliferative activity and compared this activity with that of the potent BMPs, BMP2 and BMP7. Treatment with concentrations up to 10 µM LB35 or LB57 demonstrated no toxic effects in C2C12 cells after 24 h. Interestingly, low concentrations of LB35 and LB57 (0.01 - 10 µM) showed significant increases in cell viability, ranging from 118% to 264% over control. Treatment of C2C12 cells with BMP2 or BMP7 (0.01 - 1 ng/mL) also significantly increased cell viability (120% to 182% over control). To determine whether the observed increases in cell viability reflected stimulation of proliferative activity, cell counts of LB-treated cultures were carried out and revealed significant increases in cell number following 24 h exposure to low concentrations of LB35 and LB57. Moreover, flow cytometric analysis of the cell cycle in cells treated with LB35, LB57 or BMP7 showed significant shifts in percentage of cells in S and G2/M phases. Thus, the activity profile of LB35 and LB57 appeared to mimic that of BMPs in both cell viability and flow cytometry assays. To determine whether LB35 or LB57 act through BMP receptor-dependent pathways, cell viability assays were performed in C2C12 cells in the presence of Dorsomorphin, a type I BMP receptor inhibitor. Pretreatment with Dorsomorphin abolished the increases in cell viability stimulated by either LB35 or LB57, confirming that this activity is dependent on signaling through BMP receptors. To date, none of the few identified activators of BMP signaling have been shown to be dependent on BMP receptor activity. Efforts to assess how the LB compounds activate BMP receptor-dependent signaling are currently underway. The discovery of small molecule agonists or positive allosteric modulators of BMP signaling would help to overcome the translational barriers plaguing the clinical use of BMPs and would represent a key advancement in the field of BMP receptor pharmacology.

P1422

**Dexamethasone inhibits canonical notch signaling pathway in trabecular meshwork.**

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**Purpose:** Steroid induced glaucoma (SIG) is associated with increased extracellular matrix (ECM) accumulation, resistance to aqueous humor outflow via trabecular meshwork (TM) and elevated intraocular pressure (IOP). Notch signaling pathway is an evolutionarily conserved pathway which is important in physiological processes like cell fate and differentiation decisions and pathophysiological
processes like tissue fibrosis. Here, we investigated the effects of corticosteroid hormone dexamethasone (DEX) on Notch signaling on ECM modulation in the TM. **Methods:** Primary Human TM (HTM) cells were treated with 100nM DEX in serum free media for 24 hours alone or in combination with - a) N-(N-[3,5-difluorophenacetyl]-l-alanyl)-S-phenylglycine t-butyl ester- DAPT (Notch signaling inhibitor), b) Phenethyl isothiocyanate- PEITC (Notch signaling activator), c) RU486 (Glucocorticoid receptor inhibitor) - and investigated for canonical notch signaling and ECM protein expression. Expression of Notch receptors/ligands, effector Hes, and ECM were evaluated by immunofluorescences technique, real-time quantitative PCR (qRT-PCR) and western blotting. Student’s t-test was used for statistical analyses and results were significant if p<0.05 with a sample size of n≥3 in each experiment.

**Results:** DEX significantly induced ECM proteins like COL1A (p<0.018), FN1 (p<0.024). Also, pro-fibrogenic marker α-SMA (p<0.015) was upregulated significantly by DEX. MMP2 (p<0.001) and MMP9 (p<0.000) transcripts were significantly downregulated by DEX treatment. The canonical Notch signaling mRNA and proteins were significantly downregulated by DEX compared to the control. Notch receptors like Notch1 (p<0.000), Notch2 (p<0.007), Notch3 (p<0.000), Notch4 (p<0.000) and Notch ligands including Jagged1 (p<0.018), Jagged2 (p<0.000), DLL1 (p<0.000), DLL3 (p<0.000) and DLL4 (p<0.000) were downregulated significantly in DEX treatment for 24 & 48 hours along with Hes1, a Notch downstream target, (p<0.000) which was significantly decreased. DEX mimicked the effects of DAPT in downregulating the expression of Notch1, and Hes1. On the other hand, treatment with PEITC and blocking DEX signaling by RU486 partly restored the decrease in Notch1 and Hes1, which was decreased due to DEX treated HTM cells.

**Conclusions:** Our preliminary data demonstrates that steroid like dexamethasone could potentially inhibit the Notch signaling to deregulate the ECM homeostasis. From this study we speculate that inhibition of autocrine/paracrine notch signaling can significantly modulate the expression of ECM proteins and alter their remodeling leading to an increase in IOP. Thus, activation of notch signaling pathway can be a potential target for the intervention in SIG.

P1423

**Analyzing the mechanism of Notch activation induced by immobilized ligand.**

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Notch signaling pathway is widely conserved across species and it controls cell proliferation and differentiation. Notch signaling is regulated by cell-cell contact because both Notch receptor and Notch ligand are present at the cell surface. In the process of signal transduction, Notch receptor undergoes proteolysis after binding to its ligand, releasing Notch intracellular domain (NICD) that activates target genes. In addition to receptor-ligand binding, Notch activation requires mechanical pulling force for receptor cleavage. It is thought that trans-endocytosis of Notch receptor into ligand expressing cell generates the pulling force. However, Notch signaling can also be induced by ligand coated beads where trans-endocytosis does not occur. Besides, previous reports showed that cis-endocytosis of Notch receptor is also needed for Notch transduction in a context dependent manner. Here, we hypothesized that either cell movement or receptor cis-endocytosis generates pulling force and may contribute to immobilized ligand-induced Notch activation. To test this hypothesis, we firstly focused on actin, a molecule that regulates both cell movement and endocytosis. Our data showed that Notch response to ligand coated beads was impaired when actin function in the Notch expressing cell was inhibited. We next investigated whether this signal impairment was caused by inhibition of receptor cis-endocytosis.
Inhibition of endocytosis by dynamin inhibitor did not alter the Notch response to ligand coated beads, indicating that cis-endocytosis does not contribute to Notch activation. These results suggest the possibility that the cell movement, but not receptor cis-endocytosis, provides the pulling force and activates immobilized ligand-induced Notch signaling in an actin dependent manner.

P1424

The immune response in the neural retina is not mediated by the Ig0 domain of Basigin-variant-1

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The neural retina is considered an immune privileged area, in that the eye attempts to suppress the inflammatory response to preserve vision. If there is damage to the blood-retina barrier, such as in diabetes or ocular cancer, it is possible for monocytes to travel out of the blood vessels and into the retina. While studying retinoblastoma, a research group determined that the protein, Basigin, through its extracellular domain, elicits an immune response by enhancing transcription of pro-inflammatory cytokine interleukin-6 (IL-6). Basigin is cell adhesion molecule that belongs to the immunoglobulin superfamily. The protein has two transcripts, variant-1 and variant-2, that differ in the extracellular region. The expression of Basigin variants differ, in that variant-1 is specifically expressed by photoreceptor cells in the neural retina, and variant-2 is expressed ubiquitously throughout the body, including monocytes of immune cells and Müller glial cells of the retina. Because a previous study by this laboratory indicates that the two Basigin gene products interact via their immunoglobulin (Ig) extracellular domains in the retina, the aim of the present study was to determine if the binding region in the Ig0 domain of Basigin-variant-1 is responsible for eliciting an immune response in monocytes. Thus, recombinant versions of the Basigin-variant-1 Ig0 domain were incubated with mouse monocytic RAW 264.7 cells. After 24 hours, the cell culture medium was assayed for the expression of the pro-inflammatory cytokine interleukin-6 (IL-6) via an ELISA. Cells treated with a control protein generated from the self-ligated expression vector used to make the Basigin-variant-1 recombinant proteins served as the control. The data indicates that the recombinant versions of the Basigin-variant-1 Ig0 domain do not stimulate production of IL-6 differently from the control protein. These data suggest that the Ig0 of Basigin-variant-1 does not stimulate a proinflammatory response in monocytes. The results of the current study contradict previous data that suggests the extracellular domain of Basigin-variant-1 domain induces an immune response via IL-6 production. The study provides a better understanding of the role of Basigin in the immune response in the neural retina.

P1425

Eukaryotic RND proteins Dispatched1 and Patched1 are cation-powered cholesterol transporters

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In prokaryotes, RND proteins function as proton-powered antiporters to mobilize noxious chemicals out of the cell. A transporter activity of eukaryotic RND proteins has been hypothesized, but not demonstrated. We show that the eukaryotic RND proteins Dispatched1 and Patched1, which function at different key points in the Hedgehog signaling pathway, act as cation-powered cholesterol transporters. We find that the trans-membrane sodium gradient powers Dispatched1-catalyzed release of cholesterylated Sonic hedgehog, a key step in Hedgehog signal initiation. In contrast, we find that the
trans-ciliary potassium gradient is necessary for Patched1 repression of Smoothened, a key step in Hedgehog signal reception mediated by cholesterol. We identify a set of conserved, putative, cation-coordinating residues necessary for Dispatched1 and Patched1 activity. Our results clarify the transporter activity of essential eukaryotic RND proteins, and demonstrate that the two main cation gradients of animal cells differentially power cholesterol transport at two crucial steps in the Hedgehog pathway.

P1426

Regulation of human sebaceous gland cells by heparinoids
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[Objectives] Heparin-like substances (heparinoids: HPoids) have been approved as therapeutic agents for skin diseases and are often used for the purpose of moisturizing the skin, but there are many uncertainties regarding their effects and the mechanisms involved. The purpose of this study was to analyze and identify the effects of HPoids on the function of sebaceous gland cells. [Methods] Immortalized human facial sebaceous gland cells (SZ95 cells) were cultured in the presence of various stimulants with or without HPoids. The lipid amount, gene expression of lipid synthesis-related enzymes, and proliferation of SZ95 cells were examined. Apoptosis of the cells was analyzed by Annexin staining using flow cytometry. [Results] Lipid synthesis in SZ95 cells was strongly induced by Linoleic Acid (LA) in the culture medium. Treatment of the cells with HPoids decreased the amount of lipid, and suppressed PPARγ mRNA expression. Furthermore, LA induced apoptosis in a concentration-dependent manner, which was suppressed by HPoids. HPoids suppressed cell proliferation, but did not apparently induce apoptosis even at 0.1% w/v. Gene expression of inflammatory cytokines in SZ95 cells was enhanced when cells were stimulated with lipopolysaccharide (LPS) or tetradecanoyl phorbol acetate (TPA), while only the latter case was suppressed by HPoids. [Conclusions and Discussion] HPoids suppress proliferation, lipid synthesis, and sebum release (holocrine) of SZ95 cells but do not induce apoptosis. HPoids also attenuate inflammatory cytokine expression of the cells in some context. In sum, it appears that HPoids regulate proliferation, maturation and inflammatory response of SZ95 cells. Thus, HPoids may suppress excessive sebum-evoked skin inflammation such as acne by suppressing lipogenesis and affecting multiple pathways in sebaceous gland cells.

P1427

The effect of ethanol and PTHrP on estrogen-induced proliferation of mammary epithelial cells
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Epidemiological and experimental studies have shown a link between moderate alcohol consumption and an increased risk of breast cancer in women. Several mechanisms involving alcohol-induced breast cancer have been suggested including increased estrogen induced cell proliferation. Because estrogen signaling is important during adolescent mammary development, we were interested in determining the effect of alcohol on the pubertal development of the mammary gland. Three-week old mice fed a liquid diet consisting of 0, 10 and 20% ethanol showed an increase in epithelial density and proliferation at the terminal end bud (TEB), as shown by whole mount analysis and BrdU incorporation. Mice
overexpressing PTHrP, an inhibitor of estrogen in the mammary gland, fed a liquid diet containing 20% ethanol did not show an increase in epithelial cell density or cellular proliferation at the TEB as compared to wild type mice. Together, these studies suggest that alcohol exposure during pubertal development alters the normal development of the mammary gland perhaps by interfering with estrogen signaling. To further elucidate the role of alcohol and PTHrP in mammary epithelial proliferation, we measured alcohol induced cell proliferation in the estrogen receptor (ER) positive MCF-7 cell line and in the estrogen receptor negative cell line MDA-MB-231. Ethanol treatment increased cellular proliferation only in ER positive MCF-7 cells but not ER negative MDA-MB-231 cells. Pre-treatment with PTHrP inhibited the alcohol induced proliferation of MCF-7 cells. Together these results suggest that alcohol induced cellular proliferation is due in part to the activation of estrogen receptor signaling and that this activation can be inhibited by PTHrP.

P1428

**Temporal organization of E3 ligases at the Myddosome Signaling Complexes**

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The Myddosome, composed of MyD88, IRAK4, and IRAK1/2, is a supramolecular organizing center (SMOC) that transmits biochemical signals from Toll-like receptors (TLRs) and interleukin-1 receptors (iIL1Rs). The Myddosome stimulates the formation of polyubiquitination (pUb) chains, that are critical to activate NF-κB and AP-1 transcription factors. The Myddosome activates multiple E3 ligases, such as TRAF6 and pellino1 that generate K63-pUb chains and the LUBAC complex (HOIL1, HOIP, and SHARPIN) that catalyzes M1-pUb chains. It remains unclear how the Myddosome is temporally connected with ubiquitin machinery to efficiently impart biochemical signals. In this study, we employed CRISPR/Cas9 knock-in to generate EL4 cell lines with GFP tagged MyD88, together with E3 ligases TRAF6, pellino1, or HOIL1 labelled with mScarlet. Using IL1-functionalized supported lipid bilayers and live cell TIRF microscopy, we measured the temporal organization of E3 ligases at Myddosomes. We found that IL1 stimulation triggers the recruitment and assembly of MyD88 to the plasma membrane, and these assemblies recruit multiple E3 ligases. Our data revealed that TRAF6, pellino1, and HOIL1 are Myddosome components. Computational image analysis indicated that E3 ligases colocalization correlates with the formation of stable MyD88 spots. Moreover, we found that E3 ligases have distinct recruitment dynamics with TRAF6 and pellino1 are recruited before HOIL1. Furthermore, the MyD88 assemblies localized with HOIL1 are larger, than those localized with TRAF6 and pellino1. This suggested larger MyD88 structures, possibly clusters of Myddosomes, are required to recruit HOIL1. These results demonstrated the temporal organization of the ubiquitin signaling machinery in Myddosome and IL1R inflammatory signaling.

P1429

**Distinct Roles for Notch1 and Notch3 in Human Adipose-Derived Stem/Stromal Cell Adipogenesis**

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The role of the Notch signaling pathway in adipogenesis has long been controversial as the role of individual Notch receptors appears to vary with experimental conditions. Here we demonstrate that in
human adipose-derived stem/stromal cells (hADSCs), Notch1 and Notch3 have distinct expression profiles and roles during adipogenesis. Expression of these Notch receptors changed during adipogenesis with Notch3 expressed prior to the formation of lipid vesicles and Notch1 only appearing after vesicle formation. In addition, the siRNA-mediated Notch3 knockdown demonstrated an increased expression of PPARγ, an adipogenic marker, that was paralleled by a marked decrease in expression of β-catenin, the key functional component of the canonical Wnt/β-catenin signaling pathway. This study deepens the understanding of Notch signaling by clarifying the distinct roles of Notch1 and Notch3 during adipogenesis offering a novel therapeutic target for research aimed at obesity and diabetes.

P1430

Diverse cell stimulation kinetics identify predictive signal transduction models

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Predictive understanding of signaling pathways will expand our knowledge of cellular function and dysfunction. However, predicting cell signaling responses upon environments that change over time or upon genetic mutations is currently a challenge. The reason for this predicament is that the mechanisms of how cells sense, process, and respond to extracellular stimulations are not yet fully understood. Here we present an integrated experimental and theoretical framework to perturb cells upon cell stimulations that change over time (defined as “kinetic stimulations”) and quantitatively compare predictive models of signal transduction networks. To demonstrate the feasibility of this approach, we used the High Osmolarity Glycerol (HOG) Stress Activated Protein Kinase (SAPK) signaling pathway in the yeast Saccharomyces cerevisiae model organism. We expose cells to different osmotic stimulations that change as step, pulse, linear or non-linear gradients over time to differentially regulate Hog1 dynamics. Under these conditions, we quantify Hog1 nuclear localization as a measure of signal transduction in single cells using time-lapse microscopy. Upon these distinctive osmolyte kinetic stimulations that change over time, we observe Hog1 intensity, duration, and rate of activation are differentially modulated. To understand how distinct cellular responses are achieved upon employed kinetic stimulation conditions, we established a general network inference framework and parametrized several generic signaling networks. We demonstrate that utilizing multiple diverse kinetic stimulations better constrains model parameters and enables predictions of signaling dynamics upon unforeseen biological conditions that would be impossible using traditional dose-response or individual kinetic stimulations. To demonstrate our approach, we use experimentally identified models and find that models constrained with multiple diverse kinetic cell stimulations enable us to predict signaling dynamics in normal, mutated, and drug-treated conditions upon multitudes of kinetic cell stimulations. By combining this approach with information theory criteria, we supplement intuition with optimally designed experiments that best constrain model parameters to identify predictive models. Using this approach, we found that specific proteins and reaction rates are most sensitive to specific extracellular stimulation conditions. This approach is flexible and applicable to many different pathways, in different organisms and for a range of environmental conditions. In the future, such an approach maybe applicable to predict signaling responses upon genetic mutations, altered environmental exposure or drug treatments, and identify therapeutic targets.
Spindel Microtubule Organization

P1431

Dynamic Complexity of Mitotic Spindle Microtubules

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Mitotic spindle microtubules interact with centrosomes, kinetochores, chromosome arms, each other and other cellular elements in regulating chromosome movement and cell cycle progression through M phase. Study of spindle microtubule assembly/disassembly kinetics utilizing fluorescence dissipation after photoactivation render a biphasic recovery plot distinguishing two populations, those with turnover half-time of a few minutes and those with a turnover of a few seconds. These two populations have conventionally been considered to reflect the dynamics of kinetochore versus non-kinetochore microtubules respectively. To test this and other conventions regarding mitotic spindle microtubule dynamics, we carried out photoactivation experiments while manipulating specific microtubule interactions. We first eliminated end-on microtubule kinetochore interactions by depleting the NDC80 complex in U2OS cells. In the absence of end-on interactions with kinetochores, photoactivation analyses remained biphasic with slow and fast turnover populations. Loss of kinetochores increased the percentage of fast-turnover microtubules and decreased the slow-turnover population. In addition, the half-time for recovery of the slow population was decreased. These results suggest that only a portion of slow turnover microtubules are eliminated when end-on attachment of microtubules is abrogated, leading to the conclusion that other slow turnover populations of microtubules co-exist within the spindle. We hypothesized that at least one contributor to this additional slow turnover population were interpolar microtubules. Analysis of monopolar spindles after treatment of cells with the Eg5 inhibitor, STLC, to eliminate interpolar microtubules led to a decrease in the half-time for turnover of the slow population, supporting our hypothesis. Experiments utilizing cold treatment of cells have previously shown that non-kinetochore spindle microtubules are selectively sensitive to reduced temperature. In photoactivation experiments, reduction of temperature to ~25°C increased the half times of slow and fast populations when compared to controls at 37°C, but resulted in only minor changes in proportions of each. Our studies indicate that the mitotic spindle is comprised of multiple microtubule sub-populations and that the conventional binary interpretation of photoactivation experiments as reflecting kinetochore and non-kinetochore microtubule dynamics is an oversimplification. More precise dissection of the dynamics of microtubule sub-classes are required for a full understanding of the role of mitotic spindle dynamics in facilitating chromosome movement and cell cycle control in M phase.

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Ase1/PRC1 Domains Dynamically Slow Anaphase Spindle Elongation and Recruit Bim1/EB1 to the Midzone

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Mitosis requires the complex rearrangement of the microtubule network to successfully segregate the genome. During anaphase, interdigitating antiparallel microtubules within the spindle midzone maintain
the spindle structure while elongating the spindle; however, how cells regulate microtubule crosslinking activity to control the rate and duration of spindle elongation during anaphase is poorly understood. The Ase1/PRC1 family of proteins preferentially crosslinks these antiparallel microtubules, and in this work, we test the hypothesis that Ase1/PRC1 proteins use distinct domains to slow spindle elongation rates. Using budding yeast Ase1, we identify unique roles for the spectrin and carboxy-terminal domains during different phases of spindle elongation. We utilize in vivo fluorescence microscopy to show that the spectrin domain promotes the recruitment of Ase1 to the spindle midzone before anaphase onset and slows the rate of spindle elongation most during early anaphase. This requires conserved, positively charged amino acids within the spectrin domain that are predicted to interact with the microtubule surface. In contrast, the carboxy-terminal domain of Ase1 regulates spindle elongation during late anaphase. Truncation alleles that remove a portion of the carboxy-terminal domain fail to form a stable midzone in late anaphase, produce faster elongation rates, and exhibit frequent spindle collapses. We find that the carboxy-terminal domain interacts with the plus-end tracking protein Bim1/EB1 and recruits it to the spindle midzone. Ablating this interaction with point mutations in Ase1 decreases the length of the midzone in late anaphase. Overall, our results suggest that the Ase1 spectrin and carboxy-terminal domains provide cells with a modular system to tune midzone activity and control anaphase elongation rates.

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Coordination of spindle-positioning forces during the asymmetric division of the C. elegans zygote

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In the Caenorhabditis elegans zygote, astral microtubules generate forces, pushing against and pulling from the cell periphery. They are essential to position the mitotic spindle. By measuring the dynamics of astral microtubules, we revealed the presence of two populations, residing at the cortex for 0.4 s and 1.8 s, which correspond to the pulling and pushing events, respectively. Such an experiment offered a unique opportunity to monitor both forces that position the spindle under physiological conditions and study their variations along the anteroposterior axis (space) and the mitotic progression (time). By investigating pulling-force-generating events, at the microscopic level, we first confirmed that an anteroposterior imbalance in dynein on-rate encodes the asymmetry in pulling forces. The regulation by spindle position recently proposed reinforces this asymmetry only in late-anaphase. Furthermore, we exhibited, for the first time, a direct proof that the force-generator increasing persistence to pull (processivity) accounts for the temporal control of the pulling forces throughout mitosis. We propose a three-fold control of pulling forces, by the polarity, spindle position and mitotic progression. Focusing on pushing force, we discovered a correlation between the pushing-microtubule-population density and the stability of the spindle position during metaphase, which strongly suggests that the pushing force contributes to maintaining the spindle at the cell centre. This force remains constant and symmetric along the anteroposterior axis during the division. The pulling one increases in intensity and becomes dominant at anaphase. In conclusion, the two-population assay enabled us to decipher the complex regulation of the spindle positioning during cell division.
Ultrastructural characterization of k-fiber in mitotic spindles in human cells in culture

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We are interested in a quantitative 3D analysis of microtubule (MT) organization in mitosis. For this, we are applying large-scale electron tomography of cryo-immobilized and staged spindles. Our previous study on the ultrastructure of the first mitotic spindle in *C. elegans* revealed that chromosomes are indirectly connected to the centrosomes by anchoring of kinetochore microtubules (KMTs) into the spindle network. Currently, we are analyzing KMTs in mammalian cells. In contrast to mitotic spindles in *C. elegans*, KMTs in mammalian cells are organized in k-fibers. We reconstructed mitotic spindles in HeLa Kyoto cells at metaphase and analyzed the length and 3D path of each MT in the spindle. MTs that were associated with their plus ends to kinetochores were classified as KMTs. About 16% of all MTs in the mammalian spindle (roughly 4800 MTs) were KMTs with an average length of 3.2 µm (± 1.5 µm). For each kinetochore, we counted 8 ± 1.8 attached KMTs. The minus-ends of KMTs were found within an average distance of 1.6 µm (± 1.1 µm) to the center of the centrosome. We also observed short MTs attached to the kinetochores, which were not directly associated with the centrosomes (their minus-end were further then 1 µm). In fact, only about 50% of the KMTs were in direct contact with one of the centrosomes. We also calculated the average cross-section area of each k-fiber, which was in the range of 0.06 ± 0.05 µm². However, in some cases, the diameter of the k-fibers increased close to the centrosome due to expanding distances between single KMTs. In addition, k-fibers were not only composed of KMTs but also of MTs associated with neither the kinetochores nor the centrosomes. Currently, we are working on a detailed biophysical model of k-fiber structure and function in mammalian cells.

Reciprocal regulation between cytoskeletal elements and microRNA-31 during cell division

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Proper cell division is critical for all biological systems, and its disruption is catastrophic for the organism. Cell division is dependent on the precise choreographic movements of mitotic apparatus, which is a highly dynamic structure that consists of a large complex of microtubules, proteins, and actin. We observed that miR-31 targets are involved with the regulation of actin dynamics (*Fascin, Rab35*, and *Gelsolin*). They are localized and enriched in the perinuclear region in non-dividing cells and associate with mitotic spindles in dividing cells in the sea urchin embryo. This striking oscillation of localization of miR-31 and its targets suggests that these RNAs may play a direct role in spindle assembly, and/or cell cycle progression. To determine the importance of microtubules in miR-31, its targets, and actin dynamics, we treated the embryos with microtubule disruptors (nocodazole) and observed a decrease in the localization of *miR-31* and *Fascin* in the spindle midzone. Nocodazole also caused a decrease in actin localized in the spindle midzone. In the presence of microtubule stabilizers, hexylene glycol, and Taxol, we observed *miR-31, Fascin,* and *Rab35* are further enriched at the mitotic spindles. These results suggest that microtubules regulate the localization and potentially the stability of these transcripts. Additionally, we observed more cytoplasmic actin in hexylene glycol and Taxol treated embryos,
indicating that microtubules could be important for the formation of actin. To examine the function of miR-31 during cell division, we microinjected miR-31 inhibitors into zygotes. We found that miR-31 inhibition resulted in more extensive astral microtubules and less or incorrectly directed interpolar and kinetochore microtubules in cleavage stage embryos compared to the control. These results indicate that miR-31 regulates the cytoskeleton-rich mitotic spindle structure during mitosis. Thus, the cytoskeletal microtubules regulate the localization of miR-31 and its targets, and miR-31 regulates the microtubule structures. Since proper spindle formation is a prerequisite for faithful segregation of chromosomes, understanding additional regulatory factors of cell division will be important for cancer, growth, and development.

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**Spindle chirality dynamics during mitosis**

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Equal division of the genetic material into two newly formed daughter cells is performed by the mitotic spindle, a complex micro-structure that consists of two poles, microtubule bundles extending between the poles, and a large number of associated proteins. During spindle formation, microtubule bundles adopt a ‘spindle’ shape, while navigating chromosomes to properly orientate within it. Individual microtubule bundles obtain different shapes depending on the forces acting on them, which are generated by motor proteins and microtubule dynamics. We have previously shown that the spindle shape in human cells is chiral as bundles are twisted along a left-handed helical path, and this chirality can be abolished by inactivation of the motor protein Eg5/kinesin-5 (Novak et al., Nat Commun 2018). However, it is not known how chirality changes throughout mitosis, how other microtubule-associated proteins affect it, and to what extent it is conserved. Here we show that spindles are more twisted in very late metaphase and early anaphase, compared to prometaphase and late anaphase. Furthermore, we tested additional motor and non-motor proteins that could be involved in spindle chirality. Among the candidate microtubule-associated proteins, we found that depletion of PRC1 or Kif18A/kinesin-8 leads to a decrease of left-handed twist, suggesting a role of these proteins in producing or maintaining spindle chirality. Similarly, depletion of the augmin subunits HAUS6 or HAUS8 resulted in a loss of spindle chirality, possibly due to weakened microtubule bundles. Finally, we show that spindle chirality is present in other organisms, specifically in the amoeba *Naegleria gruberi*. Although this organism has a spindle that is different from those in human somatic cells, i.e., it does not have centrosomes, microtubule bundles still adopt a helical form. Surprisingly, bundles in amoebas typically follow a right-handed helical path. In the future, it will be interesting to find out why spindle chirality is more pronounced around anaphase onset and what role it may have in chromosome segregation.

P1437

**Augmin regulates kinetochore tension and spatial arrangement of spindle microtubules by nucleating bridging fibers**

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The mitotic spindle functions as a molecular micromachine that evenly distributes chromosomes into two daughter cells during cell division. A major mechanical element of the spindle are kinetochore fibers attached to sister kinetochores on each chromosome and laterally linked by a bundle of antiparallel microtubules called the bridging fiber. Spindle microtubules are mainly nucleated at the centrosome and on the lateral surface of existing microtubules by the augmin complex. However, it is unknown how the augmin-mediated nucleation affects functionally distinct microtubule bundles and thus the architecture and forces within the spindle. Here we show, by using siRNA depletion and CRISPR knock-out of the augmin complex subunits HAUS6 and HAUS8 in human cells, that augmin is a major contributor to the nucleation of bridging microtubules. Augmin depletion resulted in a ~70% reduction of the microtubule number in bridging fibers and ~40% in kinetochore fibers, suggesting that the bridging microtubules are largely nucleated at the surface of present microtubules. In augmin-depleted cells, the interkinetochore distance decreases preferentially for kinetochores that lack a bridging fiber, independently of the thickness of their k-fibers, indicating that augmin affects forces on kinetochores largely via bridging fibers. Without augmin the number of bridging fibers decreases, with the remaining ones mostly confined to the spindle periphery with an increased overlap length. The reduced number of microtubules also results in a slower poleward flux. Our results demonstrate a critical role of augmin in the formation of bridging microtubules and proper architecture of the metaphase spindle, suggesting a model where sliding of augmin-nucleated bridging microtubules promotes poleward flux of k-fibers and thus tension on kinetochores.

P1438

In vitro reconstitution of polarised trafficking on the central spindle
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Non homogenous distribution of intracellular components is achieved through a complex interplay between cytoskeletal tracks and motor proteins. While much is known about the biophysics of motors on isolated tracks, how the complex cytoskeleton topologies found in cells, such as antiparallel microtubule (MT) overlaps, can influence the steady state distribution of cargoes is not understood molecularly. In dividing sensory organ precursor cells for example, the antiparallel MT overlap of the central spindle controls the asymmetric segregation of signalling endosomes containing fate determinants in only one daughter cell. A modest microtubule asymmetry within the antiparallel MT overlap of central spindle, with more MTs on the anterior side of the spindle, generates one order of magnitude higher (than the microtubule asymmetry) bias distribution of endosomes. How this non-linearity functions and effects polarised transport remains unknown. Here we explore the behaviour of motor proteins on asymmetric microtubule track in a minimal, simplified reconstituted system in vitro. Using multiplexed micropatterning of active proteins, we first established a model antiparallel microtubule array akin to the central spindle where both the degree of MT asymmetry and length of antiparallel overlap can be controlled. Indeed, while that soluble microtubule bundling proteins like Ase1/PRC1 are insufficient to autonomously organise the desired MT topology, micropatterning of active microtubule bundlers like Pavatori/MKLP1 is competent to organise a controlled interdigitated array of microtubules. Our approach is poised to explain at the molecular level the emerging non-linear effects of cytoskeletal asymmetry on steady state cargo distribution and can be used as such to explain polarized trafficking in other antiparallel overlaps such as dendrites in vertebrates.
Mechanics of the fission yeast spindle
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An open question in mitosis is how much force the mitotic spindle exerts to segregate chromosomes. In the fission yeast \textit{S. pombe}, the spindle can be regarded as a simple bundle of overlapping microtubules that elongates during anaphase B to push the nuclei and chromosomes apart. We have discovered conditions under which the majority of cells fail during anaphase. In these cells, chromosomes cannot fully segregate and the spindle continues to elongate adopting gradually a bent shape rather than its normal straight shape. Ultimately these spindles fail abruptly, by either breaking near the spindle midzone, or by disassociation near the pole. We hypothesize that spindles exert pushing forces to elongate and bend their microtubules into this shape. Using simple beam theory, our preliminary estimates suggest that the spindle exerts 100’s picoNewton forces to bend itself, and that similar forces are needed to break the spindle. This magnitude of force is consistent with the predicted action of 100’s of motor proteins which each provides picoNewton forces. Using spindle bending as a biosensor for anaphase forces, we are currently testing how spindle proteins contribute to force production and spindle stability. The cross linker protein Ase1 (PRC1 orthologue) plays a key role, as spindles in \textit{ase1} mutants fail to bend and readily break. In contrast, spindles in mutants of candidate anaphase motor proteins (kinesin-5 Cut7, kinesin-6 Pkl1 and kinesin-14 Klp9) still bend like those in wild type cells. Our findings in fission yeast provide quantitative force measurements and offer new insights into the molecular bases of force production and spindle stability, which will ultimately inform on the mechanics of more complex spindles such as those in animal cells.

Synthetic Cell Biology

Engineering of optogenetic signaling probes reveals a dual light and temperature sensitivity in the fungal BcLOV4 photoreceptor
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Optogenetic probes of cell signaling provide high spatial and temporal control over intracellular signals. However, current probes often consist of multiple protein components that 1) require stoichiometric tuning and 2) elevate the basal signaling levels of the pathway under study. To address these limitations, we sought to develop optogenetic probes from the recently-characterized photosensitive protein bCLOV4 from \textit{Botrytis cinerea}. bCLOV4 is a single protein that translocates from the cytoplasm to the plasma membrane when illuminated with blue light in mammalian cells. We applied bCLOV4 to stimulate Ras/Erk or PI3K/AKT signaling, two pathways that regulate cellular growth and proliferation. Surprisingly, characterization of these probes revealed a unique temperature and light sensitivity of bCLOV4 that governs the dynamics of bCLOV4 translocation and pathway stimulation. bCLOV-based probes could robustly activate both Ras/Erk (bCLOV-SOScat) and PI3K signaling (bCLOV-ISH), with lower basal (dark-state) signaling and higher fold-change induction compared to commonly used 2-component
optogenetic probes. However, sustained light stimulation of the bcLOV-based probes resulted in only transient signal activation for each pathway. We discovered that the stability of bcLOV membrane localization is highly sensitive to temperature, such that membrane bound bcLOV4 is stable at lower temperatures (30°C) but rapidly dissociates from the membrane at increased temperatures (~15 minute half-life at 40°C). The rate of bcLOV4 dissociation and signal inactivation depended on both the temperature and the light dosage used. We generated a parsimonious 3-state model of the effects of temperature and light on bcLOV4 translocation dynamics, and we fit the model to live cell imaging of bcLOV4 translocation. Our model fully predicted the dynamic behavior of our Ras and PI3K signaling probes with high temporal resolution over a range of experimental temperatures. Notably, our model predicts an irreversibly inactivated bcLOV4 state that has not yet been characterized. Future work will characterize the predicted inactive bcLOV4 state in order to rationally modulate the temperature sensitivity of bcLOV-based optogenetic probes.

P1441

**Controlled phase separation of synthetic membraneless organelles for selective cargo recruitment and cell cycle control**

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Liquid-liquid phase separation (LLPS) of intrinsically disordered proteins (IDPs) into biomolecular condensates or membraneless organelles is a fundamental organizational principle in cell biology. These structures serve to sequester or concentrate client proteins and RNAs in vivo to drive cellular biochemistry and thus, are an attractive target for materials science and bioengineering. In this work, we take advantage of the self-assembly principles of the disordered RGG domain of LAF-1 to generate synthetic condensates in vitro and in vivo. This is accomplished by triggering multimerization, thereby increasing valency, and driving LLPS by both chemical and optochemical means in vitro. Further, we extend this approach to trigger orthogonal condensate formation in living cells using *S. cerevisiae* as a model system. In order to control cell activity, we make use of highly specific protein-protein interaction motifs to selectively target and sequester cellular cargos in vivo. We demonstrate robust recruitment of the polarity factor, Cdc24 (GEF for Cdc42 GTPase) to synthetic membraneless organelles and loss of its polarized localization at the cell cortex. Importantly, this recruitment results in a functional knockdown of Cdc24 activity, leading to cell cycle arrest. Taken together, this system holds promise as a way to selectively sequester cellular factors to control or fine tune cell fates and behavior.

P1442

**A Microfluidics Approach to the High-Throughput Generation of Quantifiable Cell-Free Protein Synthesis Systems**

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Synthetic biologists construct parts, devices and systems to engineer cells for applications in medicine, biofuels, chemical commodities, and the environment. A common problem with projects that focus on the synthesis of natural and engineered proteins is unpredictable translation directed by ribosomal binding sites (RBS). Researchers have hypothesized that base pairing interactions between RBSs and downstream coding sequences form secondary structures that affect the efficiency of translation initiation. Bicistronic designs (BCDs) were developed in 2013 by Drew Endy and his colleagues to enable more predictable and reliable in vivo translation. BCDs have an RBS that leads to the production of a leader polypeptide with no functionality and a second RBS that directs the production of a protein of interest. BCDs are better at producing predictable protein levels in bacterial cells than simple RBSs, thereby improving the ability of BCDs to function reliably and predictably as classic synthetic biology parts. In a companion study, we used GFP expression to compare the translational efficiencies of 19 BCDs during cell-free protein synthesis (CFPS) to their efficiencies in vivo and found a rank correlation of 0.88. This report describes our efforts to expand the toolkit for CFPS protein production to microfluidic droplets. We used a microfluidic flow control system to rapidly produce nanoliter-scale droplets and characterized them with microscopy. Image analysis of the droplet fluorescence intensities allowed us to quantify and compare BCD-directed protein synthesis within droplets. We automated this process for many images to accelerate the workflow. We focused on three BCDs for CFPS of GFP in droplets and documented significant increases in observed fluorescence compared to batch CFPS outside of droplets. We also found that the rank order function for the three BCDs was the same in droplets as it was in batch CFPS. Our results support the use of BCDs to gain predictability of protein production in CFPS. These results set the stage for partitioning large libraries of gene regulatory combinations into microfluidic droplets to find the best combinations suited for a given synthetic biology application.

A gene signal amplifier platform for monitoring the unfolded protein response

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Gene expression in mammalian cells results from coordinated protein-driven processes guided by diverse mechanisms of regulation, including protein-protein interactions, protein localization, DNA modifications, and chromatin rearrangement. Increasingly the study of gene expression signatures and profiles is becoming the focal point in biological research, particularly in the characterization of stress-response signaling pathways. Current technologies to monitor gene expression rely on transcriptomic analysis that do not provide temporal resolution of gene expression dynamics. To address the need to monitor chromosomal gene expression generating a readily detectable signal output that recapitulates gene expression dynamics, we developed a gene signal amplifier platform that links transcriptional and post-translational regulation of a fluorescent output to the expression of a chromosomal target gene. The platform is composed of a tunable orthogonal gene network to amplify the output signal, while relying on the chromosomal integration of the main regulator using CRISPR-Cas9 to readily link the genetic circuit to a target gene, thereby easily adapting the system to monitor any cellular target. The gene signal amplifier platform was validated by developing a multiplex reporter system for monitoring markers of the unfolded protein response (UPR), a complex signal transduction pathway that remodels
gene expression in response to proteotoxic stress in the endoplasmic reticulum. A predictive mathematical model was also generated that allows adapting the gene signal amplifier for optimal detection of the expression of any cellular gene. By recapitulating the transcriptional and translational control mechanisms underlying the expression of a target gene with high sensitivity, this platform provides a novel technology for multiplex detection of mammalian gene expression that will enable characterization of gene expression signatures of physiologic and pathogenic processes.

P1444

Lentiviral transduction of tardigrade unique proteins for chronic stress tolerance studies
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Introduction: Tardigrades (“water bears”) are microscopic animals that can survive extreme stress. Tardigrade unique proteins: Damage suppressor (Dsup), Cytoplasmic Abundant Heat Soluble (CAHS), Secretory Abundant Heat Soluble (SAHS), Late Embryogenesis Abundant Mitochondrial (RvLEAM), and Mitochondrial Abundant Heat Soluble (MAHS)—derived from the species Ramazzottius varieornatus—have been shown to confer stress tolerance to human cell lines. Dsup co-localizes with DNA and provides ultraviolet radiation and reactive oxygen species tolerance in human cells. Similar work has shown CAHS localized to the cytoplasm and SAHS secreted in extracellular vesicles confers tolerance to desiccation and improves cell survival. MAHS and RvLEAM enhance HEp-2 cell tolerance to osmotic stress. However, these studies focused on short-term stress, and there is limited data on chronic stress tolerance conferred by these proteins. To address this, our objective was to create mammalian cell lines that stably express the tardigrade unique proteins described above. Methods: Briefly, plasmids with Dsup, CAHS1, CAHS3, SAHS1, SAHS2, MAHS, and RvLEAM were purchased from Addgene along with a AcGFP1-N1 control plasmid. Using restriction cloning, Dsup, MAHS, and RvLEAM genes fused with a green fluorescent protein (AcGFP1) gene were ligated into the lentiviral transfer plasmid pCDH-CMV-MCS-EF1α-puro. Using PCR and Gibson Assembly CAHS1, CAHS3, SAHS1, and SAHS2 genes were assembled into lentiviral transfer plasmid pCDH-CMV-mCherry-T2A-puro. Replication incompetent lentiviral particles were generated via triple transfection of HEK293TN cells with the appropriate transfer plasmid, pMD2.G packaging plasmid, and psPAX2 envelope plasmid. Immortalized keratinocyte cells (N/TERT1, gift of James Rheinwald), human dermal microvascular cells (HMEC1, ATCC), and adipose derived mesenchymal stem cells (ASC52TELO, ATCC) were virally transduced and selected using puromycin. Results: Confocal microscopy shows stable expression of the transgenic proteins localized to expected cellular compartments. In conclusion, we have developed a set of lentiviral vectors and demonstrated stable expression of tardigrade proteins in mammalian cells. Future work includes testing the tolerance of these cells to chronic stressors. Acknowledgements: This work was supported by the American Heart Association (19IPLOI34760636).

P1445

Coupling shared & tunable negative competition against winner-take-all resource competition via CRISPRi moieties
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Competition for cellular resources often indirectly creates unintended connections between otherwise independent genetic modules, leading to loss of modularity and impairment of intended circuit function. The undesired effects of resource competition (RC) can be controlled through global or local negative feedback loops. However, these controllers have limited tunability and scalability with increasing circuit complexity. Our previous work attempting to achieve two successive cell fate transitions via cascading bistable switches demonstrates how RC can lead to winner-take-all (WTA) dynamics that deviate from the intended behavior. Here, we attempt to remediate these issues by synthetically introducing a shared and tunable system of “negatively competitive regulation” (NCR), incorporating repressive CRISPR moieties to free up cellular resources from the “winner module” to a degree proportional to its activity. This system punishes transcriptional modules that take up more than their fair share of resources while having minimal effect on modules operating within normal activity ranges. We applied the NCR strategy to separate and cascaded dual-switch systems and demonstrated NCR’s significantly increased control efficacy over global and local negative feedback controllers. Thus, we provide an alternative strategy for controlling resource competition.

P1446

Comparing in vitro and in vivo Bicistronic Design Protein Production
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Synthetic biology takes an engineering approach to biological systems for the construction of parts, devices and systems that address challenges associated with medicine, biofuels, the environment, and more. Although the production of natural and engineered proteins is fundamental to this effort, a persistent challenge to protein production has been unreliable translation directed by a given ribosomal binding site (RBS) depending on which gene of interest is downstream of it. Based on many observations, investigators have hypothesized that the 5' UTR containing an RBS and early codons can bind to downstream coding mRNA to form base paired secondary structures that decrease the efficiency of translation initiation. In 2013, Drew Endy and colleagues developed bicistronic designs (BCDs) for predictable and reliable in vivo translation. BCDs contain two RBSs and two protein-encoding cistrons. The first RBS leads to the production of a 16 amino acid leader polypeptide with no functionality, whereas the second RBS directs the production of a protein encoded by a gene of interest. A key feature of BCDs is that the stop codon for the first cistron (TAA) shares its terminal adenine with the first base of the start codon (ATG) for the second cistron. In other words, TAATG is both a stop codon in one reading frame of cistron 1 and a start codon in a shifted reading frame for cistron 2. BCDs were found to be better at producing predictable protein levels in vivo than simple RBSs, thereby improving the ability of BCDs to function reliably and predictably as classic synthetic biology parts in bacterial cells. We wanted to learn if BCDs worked equally well for in vitro protein production during cell-free protein synthesis (CFPS). CFPS employs transcriptional and translational machinery extracted from cells producing the viral T7 RNA polymerase. We built GFP expression cassettes using 19 BCDs and tested them with CFPS. We compared the rank order of the 19 BCDs in terms of in vitro CFPS GFP production with the in vivo results published by the Endy group. We calculated a rank correlation of 0.88 between the in vitro and in vivo data, showing that BCD control of protein production is comparable in both contexts. The
significance of our work is that synthetic biologists using CFPS are well advised to employ BCDs so they can produce predictable amounts of protein for widespread applications in vitro.

P1447

**A method for gene transfer in Heterosigma akashiwo, a causative organism of harmful algal blooms**

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Sustainable Development Goals are the blueprint for creating a better and more sustainable future. These international goals, adopted by the United Nations, consist of 17 goals and 169 targets to be accomplished by 2030. Goal #14, “life below water,” primarily concerns ocean conservation. Target #14.1 includes reducing harmful algal blooms (HABs) caused by marine eutrophication. The correlations between HAB dynamics and physicochemical environmental conditions, such as temperature and water eutrophication, have been extensively studied. However, an understanding of HAB ecophysiology at the cellular and molecular levels is very limited. Such insight will provide a better understanding of HAB behavior in nature. *Heterosigma akashiwo* is a cosmopolitan phytoplankton and a causative species of HABs. It requires only seawater and sunlight to proliferate and can be cultured in the laboratory. It does not possess cell walls that often interfere with genetic manipulation methods. Development of methods for genetic manipulation and sequencing of the *H. akashiwo* genome would allow for it to be used as a model organism to better understand its biology and address the HAB problem. Here we applied a gene transfer method for mammalian cells, which also lack cell walls, to that for *H. akashiwo*. As a reporter gene, plasmids containing NanoLuc (originally from *Oplophorus gracilirostris*) were transfected using the cationic polymer PEImax. We determined a suitable promoter and codon to express the reporter. We were successful in expressing the reporter in *H. akashiwo*, which may lead it becoming a model organism. Further development, including genome editing, may help characterize *H. akashiwo*’s biology at the cellular and molecular levels. The correlations between HAB dynamics in environment and physico-chemical conditions, such as temp and water eutrophication, were extensively studies. However, understanding of HAB ecophysiology at cellular and molecular point of view is very limited. Such insight will provide better understanding of HAB behavior in nature.

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**Cell Lysate-based Cell Free Expression in GUV: Toward a Synthetic Tool of Artificial Cell with Versatile Applications**

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Cell-free protein synthesis (CFPS) based on cell lysates, which allows direct access and control of gene expression in the open space, has many advantages over proteinsynthesis in vivo. In particular, the lack of cell walls allows direct manipulation of biochemical cascades that usually occur in the cytoplasmicspace. Meanwhile, we recapitulate that when cell lysate is encapsulated inartificial cell membranes, it could be the ideal cytoplasm for artificial cell capable of protein synthesis. It was, however, difficult to implement CFPS reactions using the cell lysates due to the osmotic instability in the
confined spaces provided by liposomes. In this research, we developed and optimized the cell-lysate components and demonstrated the cell lysate-based CFPS system in a giant unilamellar vesicle (GUV). We first adopted an emulsion transfer method to prepare the cell-lysate encapsulated GUV with high encapsulation efficiency. By applying ultrasound to the vesicles, it was possible to increase the homogeneity of the encapsulated cell lysis solution, and as a result, almost all GUVs starting from recombinant DNA using GFP and RFP showed sharp green and red fluorescence, demonstrating that the fabrication of artificial cells, which are feasible to synthesize various proteins inside using a cell lysate-based expression system. We further constructed robust GUVs with lipid phase separation, which enabled GUVs to keep their shape intact against unpredictable change of osmotic pressure. In this presentation, we will present the results of artificial cells encapsulating *E. coli* extracts that synthesize different proteins from a single cell-sized liposome in response to changes in the given external conditions.

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**Molecular transport by a biological reaction-diffusion system via ATP driven diffusiophoresis**

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Spatiotemporal organization, a hallmark of living cells, is generally achieved via cargo transport by energy-dissipating NTPases based on specific protein-protein interactions. In contrast, cargo transport based on non-specific coupling remains poorly explored. Here, we found that a biological reaction-diffusion system, the *Escherichia coli* MinDE system, induces patterns and gradients of completely unrelated membrane-bound macromolecules by a purely physical mechanism: ATP-driven diffusiophoresis. Using a well-established in vitro reconstitution assay on supported lipid bilayers we show that MinDE patterns are able to spatiotemporally regulate various, functionally unrelated membrane-bound proteins. These findings implied that MinDE are able to position a much larger set of proteins in the cell than previously known. Intriguingly, the ATP-driven MinDE self-organization induced directed and active net transport of lipid-anchored proteins, establishing large-scale gradients on the membrane. To interrogate this phenomenon in more detail we employed a synthetic cargo: membrane-bound DNA origami-streptavidin nanostructures. Remarkably, by varying the number of streptavidin, we found that the extent of MinDE-driven cargo transport depends on the effective size of the cargo and leads to spatial sorting of different cargo molecules. Theoretical analysis of these data demonstrated that the diffusive fluxes of MinDE and the cargo couple via density-dependent friction. This non-specific process constitutes a diffusiophoretic mechanism, so far undescribed in a cell biology setting. It potentially represents a generic active transport mechanism in cells which could be particularly important for prokaryotes that lack specialized motor proteins and might have been prevalent in early forms of life.